THE UNIVERSITY OF ALBERTA

RADIOIODINATED BLEOMYCINS - A POSSIBLE NEW TUMOR IMAGING AGENT

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Radioiodinated Bleomycins - A Possible New Tumor Imaging Agent" submitted by John R. N. McLean in partial fulfilment of the requirements for the degree of Master of Science in Radiopharmacy.

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Date August 6/75

TO MY FAMILY

ABSTRACT

The potential utility of radioiodinated bleomycin in nuclear medicine was studied by investigating radioiodination techniques, quality control procedures and tissue distribution studies in a mouse-tumor model.

A rapid and efficient procedure for high specific activity radioiodination of bleomycin was developed using a modification of the iodine monochloride method. About 80% of the radioactive iodine present was incorporated into bleomycin within 20 minutes using an ICl:bleomycin ratio of 1:1 at a pH of 6.5-7.5. The ICl concentration and the pH were found to be critical parameters for the reaction and any deviation from optimum values resulted in decreased yields.

The chloramine-T method for the radioiodination of bleomycin was found to be unsatisfactory, producing yields of only 30%.

A technique for removing free radioiodine from the reaction mixture was developed using small columns of Dowex 1x4 anion exchange resin which reduced the radiochemical impurities in solutions of iodinated bleomycin to less than 5%.

A rapid and reproducible method, using instant thin layer chromatography (ITLC, Gelman Instrument Co., Ann Arbor, Mich.), for assaying the iodinated bleomycin solutions for radiochemical purity was adopted which allowed quantitative results to be obtained within 10 minutes.

The criteria for the utility of any newly developed or adopted techniques were based on the speed and ease with which the manipulations could be satisfactorily and reproducibly performed.

When the iodine-125 labeled bleomycins were administered intravenously into mice bearing a solid form of Ehrlich's ascites tumor, high uptake of radioactivity was observed in the tumor followed by a slow rate of clearance relative to other tissues. At 6 hours after injection maximum tumor:muscle and tumor:blood ratios of 16:1 and 8:1 occurred respectively. High levels of radioactivity were observed in the kidney throughout the period of the study.

Excretion of radioactivity from the body was relatively fast with only about 30% of the initial dose remaining at 24 hours after administration. The clearance of radioactivity from the body was resolved into a rapid component with a half-time of 0.6 days and a slow component with a half-time of about 6.2 days. These components represented about 90% and 10% of the initial dose respectively.

Tissue distributions of Indium and iodine labelled bleomycins were compared using a mouse-tumor model. The iodinated bleomycin was rapidly cleared from the body and gave optimum a tumor:tissue ration of 16:1 at 6 hours after administration whereas the ^{114m}In-bleomycin was slowly excreted, showed accumulation of radioactivity in the liver and spleen, and had optimum tumor:tissue and tumor:blood ratios of 5.5:1 and 10.8:1 respectively at 48 hours after injection.

Nuclear magnetic resonance (nmr) spectroscopy studies were done on iodinated bleomycin A_2 in an attempt to determine if iodine was being covalently incorporated into the imidazole ring of the B-hydroxyhistidine moiety of bleomycin.

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INTRODUCTION

In 1966, Umezawa and his co-workers discovered and investigated the bleomycins, a group of antibiotics that were found to have significant antitumor activity, particularly in squamous cell carcinomas and malignant lymphomas (1)(2)(3). It was subsequently shown that bleomycin had a favorable tissue distribution with early, selective and relatively high uptake in tumors (4)(5) and a rapid clearance from the body by the kidney (5). It was also established that bleomycin was a chelating agent, existing in nature associated with copper (1).

As bleomycin was becoming established experimentally as a potentially valuable antineoplastic agent, Edwards and Hayes in 1969 observed a selective accumulation of ^{67}Ga citrate in some soft tissue tumors (6). In the past, nuclear medicine researchers had directed much of their efforts towards developing safe and simple methods of tumor detection and evaluation in patients with cancer. The affinity of ^{67}Ga for lymphomas and a variety of carcinomas and sarcomas offered hope that these objectives could be possibly satisfied by a new class of radiopharmaceutical, the tumor imaging agent. This class of radiopharmaceutical, ideally, would accumulate or localize in malignant tissues in general regardless of the histological type or location of the tumor. The affinity of ^{67}Ga however, was found to vary with different histological tumor types. It became evident that although ^{67}Ga would be useful clinically, it would not be able to fulfill the requirements of an ideal general purpose tumor imaging agent (7). The limitations of ^{67}Ga led to a continued search for improved tumor imaging agents with greater specificity for malignant tissues. Interest turned towards agents with a known selective accumulation in tumors and which could be easily labeled with a suitable radionuclide. Bleomycin was found to have several properties which made it an attractive candidate for radiolabeling. First, it was known to have a favorable tissue distribution with a high and selective uptake in tumors (4)(5). Second, a major portion of the injected dose was found to be rapidly cleared in the urine and this would reduce general body background and radiation dose if a satisfactory radiolabel could be found. Finally, bleomycin was known to be a chelating agent of various cations. In nature, bleomycin was found to be chelated to copper so that simple substitutions for copper could be made with a variety of bivalent and trivalent cations. The factors which would determine the usefulness of the radionuclide complex as a tumor localizing agent would include the biological stability of the complex, the uptake and clearance of radioactivity in different tissues and the physical characteristics of the labeling nuclide. After much preliminary research for a suitable cation, ^{57}Co-bleomycin was introduced as a tumor imaging agent by Nouel in 1972 (8).

Co-57 was found to be tightly bound to bleomycin and the complex was rapidly excreted in the urine but the long physical half-life of Co-57 (270 days) made it undesireable for routine clinical use (9). To circumvent the problems associated with a long physical half-life, Merick et al. and Thakur, labeled bleomycin with ^{111}Indium, a nuclide with a half-life of

2.81 days (10)(11). However, indium, like iron, is a transition metal and when it is circulating in the blood pool it will compete with iron for binding sites on transferrin (10). Hence ionic indium is slowly excreted and tends to accumulate in marrow erythroid cells and liver (12). It was quickly observed that ^{111}In-bleomycin also gave high levels of activity in liver and bone marrow and it was implied that this was due to in vivo dissociation of the ^{111}In-bleomycin with the subsequent attachment of free In-111 to the transferrin (10)(13). It was further observed that ^{111}In-bleomycin and ionic In-111 reached their best tumor-to-nontumor ratios at the same time, about 48 hours post injection (14). In-111-bleomycin gave more satisfactory results than ionic indium but the fact that the chelate was found to be weak limited the overall utility of ^{111}In-bleomycin as a general tumor imaging agent. In spite of its shortcomings however, it has continued to generate clinical interest (15)(16)(17).

Recently, efforts have been directed towards labeling bleomycin with technetium-99m (Tc-99m), a nuclide which as ideal physical properties for external scanning (18)(19)(20). The ^{99m}Tc-bleomycin appears to suffer from instability in vivo, resulting in high background activity in the bladder, kidneys, liver, stomach and heart (18). Recently, a more efficient labeling procedure has been introduced for ^{99m}Tc-bleomycin (21). Clinical studies on this improved product have not been extensively documented.

One fact has become apparent with all of the radiolabeled bleomycins studied to date. The in vivo distribution and kinetics of the labeled bleomycin is a function of the labeling nuclide (9)(19)(20). In some way the bleomycin molecule is modified by the nuclide so that each complex demonstrates a unique set of biological properties. If this is the case, then merely incorporating a radionuclide with ideal physical properties into the bleomycin molecule may not be sufficient to produce a tumor imaging agent that is close to the ideal.

The objective of this research was to increase the tumor specificity of labeled bleomycin by first isolating the most abundant and most tumor-active fraction of the bleomycin complex and then to label this fraction covalently with a suitable radioactive isotope of iodine. The net effect of these manipulations should be: decreased background radioactivity, increased in vivo stability coupled with ideal physical properties for external detection. For clinical applications of iodine-bleomycin, iodine-123 would be the nuclide of choice because of its near ideal physical attributes but I-125 was used throughout this study for convenience. The parameters studied in this thesis relate to the chemical and biological properties of the iodinated bleomycins. The chloramine-T and the iodine monochloride (ICl) methods were used to iodinate the bleomycins and their chromatographic properties were then characterised on various solvent-adsorbent systems. The biological behavior of the iodinated bleomycins were then studied in normal mice and mice bearing solid Ehrlich's tumors. Finally, the biological properties of the iodinated bleomycins and ^{114m}In-bleomycin-A_2 were compared.

SURVEY OF THE LITERATURE

I. Bleomycin

A. History and Isolation of Bleomycin

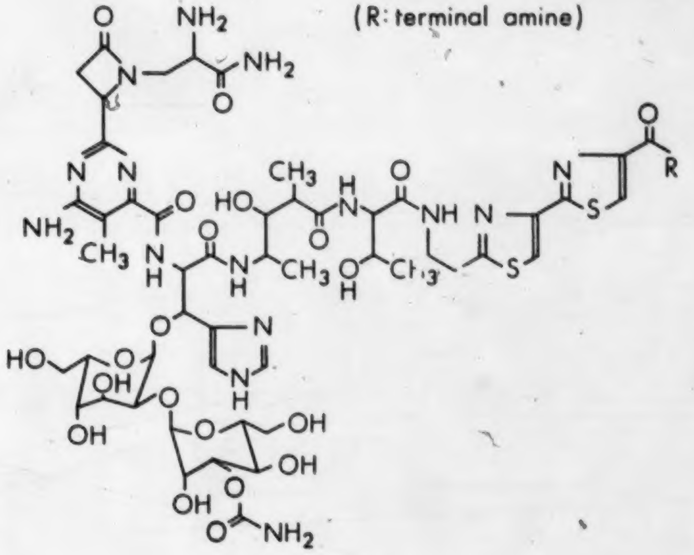
Bleomycin, the generic name for a group of water soluble basic glycopeptide antibiotics, is produced by a strain of fungus Streptomyces verticillus. It was first isolated and described by Umezawa et al. in 1966 (1). The bleomycins were extracted from culture broths by adsorption onto activated charcoal or weakly acidic cation exchange resins. The adsorbed bleomycins were eluted from the resin with water, aqueous methanol, aqueous ethanol, aqueous acetone or water saturated n-butanol at pH 4.6. The resulting eluate was neutralized and evaporated to dryness under reduced pressure and temperature. The residual grayish powder was a copper containing mixture of bleomycins.

B. Separation and Characterization of the Bleomycins

The crude mixture of bleomycins was differentiated into 2 fractions, A and B, by gel filtration chromatography on sephadex G-25 columns (2). The A components are eluted first and gave Rf values on paper chromatograms of 0.88 to 0.99. The B components are eluted last and have Rf values of 0.70 on paper chromatograms. The developing solvent was 10% aqueous ammonium chloride. The A and B fractions could be further resolved into 10 and 7 components respectively when the crude mixture was subjected to CM sephadex C-25 column chromatography using gradient elution with ammonium formate. The components have been designated as A_1, A_2, A_2'-a, A_2'-b, A_2'-c, demethyl-A_2, A_3, A_4, A_5, A_6, B_1, B_1', B_2, B_3, B_4, B_5 and B_6 (2)(3).

All bleomycin A components gave a negative Sakaguchi reaction

STRUCTURE OF BLEOMYCIN
(R: terminal amine)



TERMINAL AMINES OF SOME IMPORTANT BLEOMYCINS

A_1 •NH $-CH_2-CH_2-CH_2-SO-CH_3$

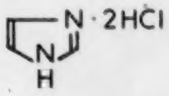
demethyl-A_2 •NH $-CH_2-CH_2-CH_2-\overset{+}{S}-CH_3$

A_2 •NH $-CH_2-CH_2-CH_2-\overset{+}{S}-(CH_3)_2X^-$

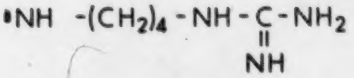
A'_2-a •NH $-CH_2-CH_2-CH_2-CH_2-NH_2 \cdot 2HCl$

A'_2-b •NH $-CH_2-CH_2-CH_2-NH_2 \cdot 2HCl$

A'_2-c •NH $-CH_2-CH_2$



A_5 •NH $-(CH_2)_3-NH-(CH_2)_4-NH_2$



B_2 •NH $-(CH_2)_4-NH-\underset{\underset{NH}{\|}}{C}-NH_2$

Figure 1

whereas all B components gave a positive reaction with Sakaguchi and similar guanidino detecting reagents (1).

The bleomycins are stable in acid and alkaline media. They are freely soluble in water, soluble in methanol, slightly soluble in acetone, ethyl-acetate, butylacetate and diethylether. These solubility characteristics make it impractical to transfer bleomycin from aqueous to organic solvent systems directly. The accepted structure for the basic bleomycin molecule is shown in figure 1 (22). The bleomycin molecule has basic functions with pKa values at 7.3, 4.7 and 2.9. The guanidino group of the B components is found to be out of titratable region and is presumed to have a pKa greater than 11.5. The alpha-amino group of the beta-amino-alanine is free and is assigned pKa 7.3. The basic function at pKa 4.7 is assigned to the imidazole because of the chemical shift of the imidazole C-2 proton which is sensitive to pH change around this pKa value. The 4-aminopyridine moeity is free and accounts for the weak base at pKa 2.9 (22).

One of the acid hydrolysis products that is characteristic of all bleomycins is a beta-hydroxyhistidine. The nmr spectrum of this component taken in D_2O using tetramethylsilane as external reference (delta=o) indicates that the doublet at delta 9.16 can be assigned to the C-2 proton of the imidazole ring (23). All bleomycins differ from one another in their terminal amine group (24).

In nature, the bleomycins are chelated to copper. The copper is removed from the molecule by treatment with 8-hydroxyquinoline. The copper free and copper containing bleomycins are biologically active. Only the copper free variety is used clinically. The characteristic Rf and Rm values for the various bleomycins have been tabulated by Umezawa *et al*. and Fujii *et al*. (21)(25).

C. Action of the Bleomycins

The bleomycins exhibit activity against a wide spectrum of bacterial cells and transplanted animal tumors. Significant growth inhibition has been demonstrated against Ehrlich's carcinoma and sarcoma 180 cells in mice (4). The antitumor effect of each bleomycin was tested on Swiss mice which had received intraperitoneal injections of 2 million Ehrlich's ascites tumor cells. Upon administration, the A_3 and A_5 bleomycin components demonstrated a marked prolongation of the mean mouse survival times in doses as low as 12.5 mcg/mouse and 6.25 mcg/mouse respectively. In contrast, the more abundant component, A_2 required 250 mcg/mouse to demonstrate a similar prolongation of survival time. Bleomycin B_2 demonstrated a significant prolongation of mean mouse survival times at 62.5 mcg/mouse (5). Bleomycin A was found to have higher antitumor activity than the bleomycin B fractions (5).

At the subcellular level, bleomycin caused single strand scission of DNA *in vitro* and *in vivo* and DNA in sensitive cells was highly damaged compared to DNA in insensitive cells (26)(27)(28)(29). In these studies, bleomycin was found to inhibit the progression of cells through the premitotic (G2) and mitotic (M) phases of the cell cycle (30). Bleomycin was also found to inhibit the incorporation of thymidine into DNA in synchronized HELA cells, Ehrlich's cells and *E.coli* cells. Recently, it has been shown that bleomycin, in low concentration, can prevent cell division without disrupting DNA synthesis (31). Therefore, single strand scission alone was not sufficient to disrupt cell division. It has been suggested that the bleomycin may act on the DNA complex as well as on other nuclear proteins inducing not only destruction of DNA but also of the mitotic apparatus such as spindles and centrioles (31).

In any event, these interactions, in vitro at least, are promoted by sulfhydryl compounds, hydrogen peroxide and ascorbic acid. In one study bleomycin A_2 was found to demonstrate a preferential binding to single strand DNA over duplex DNA (8). In vitro, 2-mercaptoethanol was used as the sulfhydryl compound. The bleomycin was bound to DNA regardless of the presence or absence of 2-mercaptoethanol. Strand scission, however, occurred only if the 2-mercaptoethanol was present (8).

D. Toxicity of the Bleomycins

1. Sub-acute Toxicity to Mice

The grade and frequency of toxic symptoms was found to be closely related to dose levels and injection periods (6). In groups of mice with daily intraperitoneal injections of bleomycin of 5, 10, 15, and 20 mg/kg/day, dirty hair and slight pilo-erection appeared 4 to 9 days after initial injection and persisted throughout the injection period. Backbone deformation and salivation appeared on days 7 to 11 and also persisted until injection regimen was terminated. Nail deformation with peripheral oozing appeared on day 9 to 11 and likewise persisted throughout the injection period.

A group of mice treated with 1 mg/kg/day showed no toxic symptoms and continued growth in parallel with control mice (6).

2. Acute Toxicity to Mice

The intravenous (I.V.) injection of 400 mg/kg and 250 mg/kg to mice caused death in all mice in 5 days. The I.V. injection of 125 mg/kg caused death in 20% of the mice within 5 days. A treatment regimen of 62.5 mg/kg I.V. allowed all mice to survive and there was no decrease in body weight after 5 days from injection. Similar results were reported for intraperitoneal and subcutaneous administration (5).

3. Toxicity to Humans

Clinical manifestation of toxicity to daily dosage regimens of 0.25 mg/kg body weight were skin ulcerations which appeared initially as erythema followed in 2 to 3 days by shallow ulceration and finally deeper ulceration as medication was continued (32). Mucosal lesions were mainly buccal, laryngeal or esophageal and occurred within 7 to 9 days from initiation of daily therapy. Anorexia, nausea, vomiting and weight loss were also observed in patients. These symptoms were mild and their frequency and intensity were dose related. Fatigue, fever and chills were observed in some patients 2 to 6 hours after administration and subsequently lasting 4 to 12 hours. Mild and severe shaking was observed in some patients within one-half to three hours after administration and lasted for up to two hours (32). The above complication occurred less frequently at doses of 0.1 mg/kg.

Rash and pruritis could be noted in 11% and 9% of patients, respectively. Shortness of breath and severe cough occurred in 6% of patients after a single administered dose of bleomycin. A generalized headache beginning 2 to 8 hours after each dose and lasting 8 to 24 hours occurred in about 10% of patients. Moderate to severe alopecia occurred in about 30% of patients treated. Hair loss was dose related and developed in nearly 100% of patients treated at higher dosage regimens. Thickened dry skin and nail changes were also noted.

The most serious adverse reaction to bleomycin was pulmonary toxicity. There was a decrease in lung capacity and vital capacity in about one-third of patients. The pathological picture associated with bleomycin therapy was that of interstitial pulmonary fibrosis and alveolar squamous metaplasis and hyalinization.

Hematological studies indicated that leukopenia occurred in about 29% of patients with an average nadir at 11.9 days and an average return to pretreatment levels at 16.5 days. Thromocytopenia was observed in 39% of patients. Slight hemoglobin depression occurred in 58% of patients. No changes were found in serum proteins or in clotting profiles. There have been no cases of severe hepatotoxicity reported (32).

E. Clinical Uses of Bleomycin

The commercially available preparations are mixtures of the copper-free bleomycins. A typical batch contains: A_2-48%; B_2-27%; A_1-2%; A_{2-}', A5, B_{1-} all less than 4% (7).

Bleomycin is currently used as a palliative treatment and/or adjuvant to surgery and ratiation therapy. Bleomycin is used in the management of: Squamous cell carcinoma of the head and neck, including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva and epiglottis, skin, larynx, paralaynx, penis, cervix and vulva, lymphomas, such as Hodgkin's and reticular cell sarcoma, testicular carcinoma, such as embryonal cell sarcoma, chorio-carcinoma and teratocarcinoma (33)(34).

F. Biological Studies of Bleomycin

1. Absorption and Excretion of Bleomycin

Bleomycin is rapidly taken up into blood from the site of injection and rapidly partitioned to the body tissues (7). High concentrations of bleomycin can be detected in blood 10 minutes after subcutaneous injection. In a study by Ishizuka, mice were injected subcutaneously with 50 mg/kg of bleomycin A complex (5). Blood levels reached a maximum in 30 minutes and fell to less than half of this maximum value at 2 hours after injection. Only trace quantities of bleomycin was detected at 4 hours post injection. Urine levels peaked at one hour after injection and then slowly decreased until only a trace (one mcg/ml) was detectable at 24 hours. About half of the injected dose was cleared through the kidneys within 4 hours of injection (5). The concentration of bleomycin in this study was determined by the cylinder plate bioassay method using B. subtilis as the test organixm (5).

2. Tissue Distribution of Bleomycin

Two types of assay methods have been used to study the tissue concentrations of bleomycin. The bioassay, or cylinder plate procedure, uses a test organism with a quantifiable sensitivity to the antibiotic. The bioassay method reflects the concentration of the antibiotic that is extractable from the tissues in active form. A tissue distribution study using a mixture of the A bleomycins was carried out in groups of normal Swiss mice and Swiss mice bearing solid Ehrlich's tumors (5)(6). Tumor mice were produced by injecting about 2 million Ehrlich's ascites tumor cells subcutaneously into the femoral region of the right leg. The tumor was cultured in the mice for 10 days before the bleomycin was administered. High concentrations of bleomycin was detected in the lung, kidney, skin and tumor. Bleomycin remained in the lung at higher concentrations than in the kidney or blood at 3 hours after injection (5). The concentration of bleomycin was higher in the tumor than in the liver, muscle, testes or spleen at 3 hours after administration. The results obtained in normal mice were not found to be significantly different from results obtained for tumor bearing mice (6).

An alternative approach to the study of bleomycin tissue distribution used tritiated bleomycin complex, which is composed mainly of A_2 (48%) and B_2 (27%) (7). The radioactive bleomycin complex was injected subcutaneously into normal mice. The mice were sacrificed after 1 hour and the distribution of radioactivity was determined. High concentrations of bleomycin was detected in skin, kidneys, testes, peritoneum and urine (7). The tissue concentration of bleomycin using the radioactive tracer technique was always found to be higher than the concentrations as determined by the bioassay or cylinder plate technique. Contrary to other organs however, the amount of bleomycin in the blood shown by the cylinder plate method was higher than the concentration shown by the radioactive method. These results suggested that all organs to varying degrees contained a bleomycin inactivating substance. The content of this substance was significantly lower in squamous cell carcinoma in mouse skin than in other tissues (7).

G. Chemistry of Bleomycin: Chelation Chemistry

The tumor specificity of ^{67}Ga citrate and its clinical usefulness to cancer diagnosis led to a continued search for substances with even greater affinity for tumors. Research efforts were directed towards radiolabeling tumor specific chemotherapeutic agents. Bleomycin had established antitumor activity and favorable biological properties. Like most antibiotics, bleomycin was also a chelating agent (1). The structural components of the bleomycin molecule had a number of sites where coordinate bonds could be formed with metal cations (22). The mechanism of chelate bond formation between the reactive species is beyond the scope of this thesis. However, a discussion of the general nature of the chelate bond may be useful in providing an insight into the approaches that have been taken in the radiolabeling of bleomycin.

1. Electron Donors and Acceptors in Chelation

The phenomenon of chelation has been reviewed by Ahrland (35) in general and Sundberg (36) specifically as it relates to histidine and the transition metals. When the water molecules surrounding a metal ion are replaced by other molecules or ions, the resulting substance is said to be a metal complex or a metal coordination compound. The group which combines with the metal ion is called a ligand. The type of bonding between the metal ion and the ligand can vary from one that is primarily electrostatic in nature to one that is predominantly covalent (35). The function of the ligand is always as an electron donor to the metal. A chelate is a metal complex in which there is more than one donor atom, and these donors are attached, directly or indirectly to each other and to the metal. Thus the metal becomes part of a ring system (36).

Donor substances can be grouped in two general categories (36). The first category includes alkenes, alkynes, aromatic hydrocarbons and their substitution products. These are classed as π donors, that is, the electrons that are available for sharing are those contained in π molecular orbitals and the adducts which they form are called π complexes. They can be described alternately as "outer" complexes since the acceptor atom does not penetrate deeply into the π orbital of the donor (37).

The second major class of donors encompasses a large group of substances in which there are non-bonded electrons (lone pairs) available for coordination (37). These are called n-donors and they include such groups as the oxygen of hydroxyl and carbonyl groups, iodine and sulfur of the organic iodides and sulfides, and nitrogen bases--in which the lone pairs are located in the atomic orbital of the respective atoms.

The various acceptor and donor classes have been characterized on the basis of their degree of polarizability which has a pronounced influence upon complex formation. Those groups with a high degree of polarizability are classed as "soft" while those with poor polarizability are classed as "hard." "Soft" metal acceptors possess large numbers of d-electrons in their outer shells which can be easily dislocated or polarized. These "soft" acceptors tend to form stable chelates with "soft" donors (35).

Acceptors which are classed as "hard" are not readily polarizable and prefer to form complexes with donors that are "hard". A typically "hard" acceptor is always characterized by high charge and/or small radius. Consequently, the hardness of an acceptor will tend to increase as its oxidation state increases (35)(37). The difference in chemical behavior characterized as "hard" and "soft" is due to differing nature of the bonds formed between donor and acceptor. Bonds formed between "hard" acceptors and donors are more electostatic, or ionic, in nature whereas bonds formed between "soft" acceptors and donors are more covalent. The character of the two bonding patterns depends on the nature of the solvent system. The electrostatic interaction between any two charged particles is stronger in a medium with a low dielectric constant. Water has a high dielectric constant and aqueous solutions therefore represent an extreme of low electrostatic interaction (35). A bond formed by a certain acceptor-donor pair will tend to become more electrostatic in character in non-aqueous solvent systems. The dielectric constant indicates that the molecules of a solvent are strongly polar and they will interact with ions and other dipoles present. This interaction is especially strong with metal cation acceptors, as they are generally small and often high in charge. Metal ions tend to be extensively solvated in all solvents of high dielectric constant (35). Thus, the solvent molecules act as ligands in competition with other ligands present in solution. The interaction between solvent dipoles and other ligands is relatively weak as the typical ligand is either an anion with a rather large radius and often with low charge, or a neutral molecule. The fluoride ion and the sulfate ion are exceptions to this rule (36). Thus, electrostatic forces in aqueous medium are minimized, while the tendency for covalent bond formation, which is responsible for "soft" behavior, will remain, as it is essentially a property linked to the electronic configuration of the acceptor. In summary then, for the formation of a complex between a hard donor and acceptor, strong bonds between these and the water molecules of their hydration shells have to be broken. This takes much energy which is not completely regained by formation of the predominantly electrostatic acceptor-donor bond. The net reaction tends to be endothermic. The resulting liberation of several water molecules from the hydration shells implies a large gain in entropy which constitutes the driving force of the reaction between hard particles (35).

Soft donors and acceptors interact only weakly with water dipoles. They are not extensively hydrated. The complex formation will not imply any large liberation of water molecules and hence no large entropy gain. The formation of a covalent acceptor to donor bond however is accompanied by a large evolution of heat which constitutes the driving force for this type of reaction (35).

Experimentally, it is found that hard acceptors show a strong preference for oxygen donors--thus oxygen is classed as a "hard" donor. Nitrogen donors form complexes in aqueous solution with most soft and medium hard acceptors such as Co^{+3}, Zn^{+2} but not with very hard acceptors. Nitrogen therefore is classed as a "soft" acceptor. A mixed oxygen-nitrogen donor such as a peptide, should combine the affinities of oxygen and nitrogen donors and should thus form strong complexes with a large variety of acceptors (38)(39).

Organic suflides can also act as ligands. Sulfides are strongly basic and hence like nitrogen, can be classified as "soft". Highly stable complexes are formed between sulfides and acceptors classified as both "Hard" and "soft". "Hard" acceptors such as Fe^{+2}, Co^{+3}, Ni^{+2}, Cu^{+2} and Zn^{+2} can form stable complexes with sulfides (35).

2. Imidazole and Metal Interactions

The imidazole molecule is classed as an aromatic heterocycle. It is structurally related to both pyridine and pyrrole (40). The so-called pyrrole nitrogen has 2 electrons in an unhybridized P-orbital while the pyridine nitrogen has a lone, nonbonded, pair in a hybrid orbital and a single electron in a P-orbital (41). The coordination site on imidazole can be recognized through consideration of the molecule's aromaticity. The pair of electrons on the pyridine nitrogen can be described as being unshared. The π electrons of the pyrrole nitrogen are a part of the aromatic sextet and any bonding to this position would compromise the aromaticity of the molecule (36) (40).

In aqueous acidic solutions imidazole will undergo protonation at the pyridine nitrogen to give the imidazolium cation with one coordination site (36).

The ylide formed by the deprotonation of the imidazole cation at Carbon-2 can play a significant role in the chemistry of the imidazole group. The most stable ylide is formed by the deprotonation at a carbon which is bonded to two positively charged nitrogens (36). This is another coordination site for cationic imidazolium. Imidazole possess two properties, basicity and π electron acceptor capabilities. Thus the ring system tends to be amphoteric being a moderately strong organic base capable of accepting positively charged species at the pyridine nitrogen as well as a very weak acid capable of loosing protons from the pyrrole nitrogen. In solutions near neutrality the unprotonated imidazole usually functions as a ligand by the unshared pair of electrons on the pyridine nitrogen (36)(37)(38). In acidic media the ylide becomes important while in basic media the anion form becomes the important chelating species (36).

3. Bleomycin as a Chelating Agent

Bleomycin has been labeled with a large variety of metal cations (42). Renault *et al*. has presented data showing that each mg of bleomycin possessed a binding capacity of 26mcg of copper (Cu^{+2}), 25mcg of zinc (Zn^{+2}), 17mcg of cobalt (Co^{+3}), 15mcg of nickel (Ni^{+2}) and 9.5mcg of mercury (Hg^{+2}). Iron and all other metals tested by Renault had a binding capacity of less than 1mcg per mg of bleomycin (42).

The factor limiting the usefulness of most of the bleomycin-metal complexes has been their instability *in vivo* (9)(10)(11). Only the bleomycin complexes of copper (43)(44), indium (10), cobalt (8) (45), platinum (46) and most recently technetium (20) have been found to be stable enough to withstand rigorous biological testing in animals and humans.

It is apparent from the previous discussions on chelation that bleomycin has a number of potential coordination sites. The reaction conditions to which the bleomycin and the metal cations are subjected will determine which ligand groups the metal will use preferentially as electron donors. This will determine not only the stability of the complex but also to what extent the radionuclide will modify the biological properties of the bleomycin (35)(36)(37).

4. Chemistry of Radiolabeled Bleomycins

A brief survey of the methods that have been used to label bleomycin with cations indicates that the procedure for divalent and trivalent metals are different (9)(11). The following is a general summary of these labeling methods. No attempt has been made to determine if these represent optimum conditions for labeling. For some metals these reaction conditions will produce stable bleomycin complexes (9)(10)(11), while for the other metals such as gallium, zinc and mercury stable complexes were not produced (42).

a. Labeling Procedures

(i) Bivalent metal cations

The radionuclides of bivalent elements are chelated to bleomycin by mixing the two solutions together at room temperature. The reaction can take place in neutral or acidic solution (10)(11)(42)(43).

(ii) Trivalent Metal Cations

The radionuclides of trivalent metals will not chelate to any great extent with bleomycin under reaction conditions that are suitable for bivalent metals. For example, under the conditions for bivalent metals, 1 mg of bleomycin will bind 0.76 mcg of indium whereas when the reaction conditions are modified, as described below, the binding capacity of bleomycin for ^{111}In increases to 80 mcg (11). For trivalent metals, the radionuclide and bleomycin solutions must be both adjusted to pH 1.5 before mixing. The solutions are then mixed together and then the pH of the mixture is slowly adjusted to 6.5 - 7.0 (9)(10)(11). Whenever a chelating agent binds to a metal, there must be a definite molar ratio between the two if complex formation is to be complete (10). It has been found empirically that 5 mg of bleomycin in a 5 ml reaction mixture is the minimum quantity that will give adequate labeling with 2 mCi $^{111}InCl_3$ (14).

b. Labeling Procedures for Technetium-99m

(i) Stannous Ascorbate Method

Fifteen mg-equivalents of bleomycin was dissolved in 5-10 ml of pertechnetate solution. To this solution was added 150-250 mg of prepared stannous chloride ($SnCl_2 \cdot 2H_2O$) in 1 N HCl. The pH was adjusted to 2.5-3.0 and after 5 minutes of constant stirring the reaction was terminated by the addition of 1-3 of ascorbic acid. The pH was then adjusted to 7.0 (20).

(ii) Stannous Pyrophosphate Method

Fifteen mg of bleomycin was dissolved in 15-30mCi of pertechnetate solution. Then 4-11 mcg of stannous pyrophosphate was added to the mixture. No pH adjustment was required and no purification steps were needed (21).

5. Chromatography of Radiolabeled Bleomycins

On paper chromatograms developed with 10% aqueous NH_4Cl, A_2 was found to have an Rf of 0.88-0.94 while B_2 had an Rf of 0.66-0.70 (4). On silica gel thin layer chromatograms developed with 10% $NH_4OOCCH_3:CH_3OH$ (1:1), A_2 had an Rf of 0.40 while B_2 had an Rf of 0.68. Radiolabeled bleomycins have Rf values that correspond to the unlabeled bleomycins (9)(10)(11)(20)(43). Paper chromatograms for determining free pertechnate can be developed with 85% methanol. With this system, labeled bleomycin will stay at the origin while free Pertechnetate will move to Rf 0.6-0.7 (21).

Thin layer chromatography using alumina as adsorbent and 10% $NH_4OOCH_3:CH_3OH(1:1)$ as developing solvent will separate reduced pertechnetate, Rf 0.08; ^{99m}Tc-bleomycin, Rf 0.06-0.7; and free pertechnetate, Rf 0.8 (21). Indium-111 chloride at pH 7.0 has an Rf of 0.00 to 0.08 on either paper or TLC silica gel using 10% NH_4Cl or 10% $NH_4OOCH_3:CH_3OH(1:1)$ solvent systems respectively (11). Under neutral conditions, colloidal insoluble $^{111}In(OH)_3$ was formed while at pH 1.5 $^{111}InCl_3$ migrated with the solvent front (11). A similar reaction pattern was observed for gallium. Cobalt-57 chloride was observed as a fast moving peak that ran to the solvent front and did not interfere with A_2 and B_2 Rf values on silica gel tlc and paper chromatograms (9).

6. Stability of Radiolabeled Bleomycins

a. Indium-111 Bleomycin

The thermal stability of ^{111}In-bleomycin as prepared above was found to be excellent. The product was incubated at 37°C for 48 hours and autoclaved for 30 minutes at 121°C and 15 psi. There was no reported loss of label (11)(14). The chemical stability of ^{111}In-bleomycin was challenged in vitro by incubating the radiopharmaceutical with excess Ca^{+2} ions and Cu^{+2} ions at room temperature. Indium-111-bleomycin was found to be stable in the presence of Ca^{+2} but was unstable towards Cu^{+2}. The ^{111}In-bleomycin label was completely destroyed in 1 hour by the presence of Cu^{+2}. This suggests the possibility that ^{111}In-bleomycin may dissociate in vivo when Cu^{+2} is encountered (1). This would not be unexpected since bleomycin is known to form very stable chelates with copper (1).

b. Cobalt-57-Bleomycin

This complex could be autoclaved without any apparent instability. It has had extensive use in animal studies without any apparent dissociation of the label (11).

H. Biological Studies on Radiolabeled Bleomycin

1. Animal Studies

a. Cobalt-57-Bleomycin

The distribution of ^{57}Co-bleomycin has been studied in Swiss mice bearing solid Ehrlich's tumor in their femoral region (9)(45). Tumors were allowed to develop for 10 days before distribution studies were initiated. Co-57-bleomycin was rapidly cleared from the blood with 0.54% dose gm^{-1} at 1 hour post injection falling to 0.02% dose

gm^{-1} at 4 hours. The rate of blood clearance was not found to be dose dependent (9)(45). Strong uptake of ^{57}Co-bleomycin was found in the tumor and muscle, 3.73 and 2.31 % dose gm^{-1} respectively at 1 hour post injection. The radioactivity in the tumor was found to decrease more slowly than the radioactivity in the muscle and at 4 and 24 hours post injection the % dose gm^{-1} was 1.34 and 0.72 for tumor and 0.06 and 0.03 for muscle (9). Tumor to blood and tumor to muscle ratios reached near maximum values at 4 hours post injection with a definite maximum evident at 24 hours (45).

Individual bleomycin fractions A_2, A_1, demethyl A_2 and B_2 were isolated and labeled with Co-57 (47). The biological distribution in rats bearing tumors showed significantly higher concentrations in tumors at 2 hours for fractions A_2 and B_2. The other fractions studied did not reach as high a concentration in tumors. The tumor to blood ratio for the A_2 and B_2 fractions were not significantly different from the tumor to blood ratio attained by the bleomycin mixture suggesting that the concentration of the bleomycin in the tumor was related to the blood concentration (47). Maximum tumor to blood ratios of 31:1 were achieved at 24 hours (47).

The tumor uptake of ^{57}Co-bleomycin was compared to the uptakes attained with ^{67}Ga-bleomycin and ^{111}In-bleomycin (9)(45). Absolute tumor uptake of the 3 labeled bleomycins were found to be about equal for solid Ehrlich's tumor in Swiss mice. Therefore, the tumor to blood and tumor to tissue ratios were superior for ^{57}Co-bleomycin at 24 hours (45).

b. Indium-111-Bleomycin

The kinetics of ^{111}In-bleomycin has been studied in 3 mouse tumor models involving transplantable tumors of lung, skin and bone origin (14). Two parallel groups of ARK mice bearing the Ridgeway osteogenic sarcoma, C57B/6 mice bearing Lewis lung tumor and B-16 melanoma implanted subcutaneously 1-2 weeks before tissue studies, were injected with ^{111}In-bleomycin and $^{111}InCl_3$. The mice were serially sacrificed at 1, 6, 24 and 48 hours. The $^{111}InCl_3$ tumor concentration was greater than that of ^{111}In-bleomycin. The $^{111}InCl_3$ however was found to be cleared more slowly from the blood. The tumor to blood ratio for both agents were greated than 9:1 at 48 hours post injection (14). The ratios of tumor to non-tumor tissues were very similar for both agents suggesting that ^{111}In-bleomycin may dissociate *in vivo*. This would tend to confirm findings by Thakur *et al*. which showed that the ^{111}In of ^{111}In-bleomycin is found on transferrin within 4 hours *post* injection in man (10). Thakur has suggested that there might be competition by transferrin for the ^{111}In label and/or exchange of serum cations for the chelated indium (10). The highest ratios of tumor to blood occurred at 48 hours in all three tumor models.

This study was in partial agreement with that of Umezawa (5) on the distribution of unlabeled bleomycin in mice bearing Ehrlich's tumor. Both studies indicated that tumor concentrations of the radiolabeled and non-labeled bleomycin were at a maximum 1 hour *post* injection. The concentration of ^{111}In-bleomycin in many tissues at 1 hour however was close to that of the tumor level and this was at variance with the data presented by Umezawa. These facts, together with the studies presented for ^{57}Co and ^{67}Ga bleomycin suggest that the metabolism of bleomycin was profoundly affected by the character of the labeling nuclide (9).

c. Technetium-99m-Bleomycin

Many investigators have shown interest in labeling bleomycin with Technetium-99m (^{99m}Tc) because of the highly favorable physical characteristics of this nuclide. Early attempts at labeling proved to be unsatisfactory and poor in vivo stability was observed (19). Later attempts modified the stannous chloride labeling procedure until good yields and better in vivo stability of the product was obtained (18)(21). One study (18), used Balb/C mice bearing a transplanted KHJJ carcinoma in the flank. Tissues studies were conducted at 1.5 and 6.0 hours post injection. High ^{99m}Tc activity was observed in the kidneys at all time intervals studied. Concentration of activity in the tumor was high relative to muscle and brain and low relative to activity in the liver, spleen, stomach, lungs and skin. Tumor to blood ratios increased from 1.7 after 1.5 hours to nearly 3.0 after 6.0 hours. The mice were found to excrete 80% of the administered dose in 6 hours and 88% of the dose in 24 hours.

A second study (20) used mice of the A-Jackson strain bearing a transplantable fibrosarcoma MCS approximately 1 cm in diameter in the shoulder region. Mice were serially sacrificed at 30 minutes, 1, 2, 3, 4, 6, 12 and 24 hours. High initial uptake of radioactivity was observed in the kidney, gut, tumor, liver and stomach. Rapid declines in radioactivity were observed in blood, lung, stomach, spleen and liver. Concentration of radioactivity in the tumor declined less rapidly and maintained a constant level from 3 to 24 hours post injection (20). In tumor, 1% of the dose localized in 30 minutes and 0.58% was retained after 24 hours (20). The slow loss of activity in the tumor was associated with a 9-fold rise in tumor to blood levels over the interval of 30 minutes to 24 hours post injection. The study also noted that Tc-99m-bleomycin lost most of its antimicrobial activity but retained its ability to concentrate in various kinds of malignant tumors (21).

d. Platinum-195m-Bleomycin

Tissue distribution studies were done with mice bearing Ehrlich's ascites or Lewis lung carcinomas (46). It was found that the ^{195m}Pt-bleomycin complex gave tumor:non-tumor ratios similar to those obtained with ^{111}In-bleomycin (46). However, the platinum complexes were found to be very stable in vivo and were excreted unmetabolized. High blood activity (0.50% dose gm^{-1}) was found at 48 hours post injection (46).

2. Clinical Studies on Radiolabeled Bleomycins

a. Co-57-Bleomycin

In spite of its unfavorable half life, ^{57}Co-bleomycin has continued to generate interest clinically. A 66% accuracy of diagnosis was found in one study involving humans with known tumors (48). The rate of accuracy by tumor type was found to be 75% for lung cancers, 88% for squamous cell carcinomas and 100% in anaplastic cancers (48). Sequential profile scans showed high activity in the kidneys and bladder immediately after injection. The total counts decreased to 1/2 of the initial counts at 5 hours post injection and to about 1/20 at 24 hours, indicating a biological half-life of about 5 hours (48). The positive tumor detection rate of 66% was found to be slightly inferior to the detection rate for ^{67}Ga-citrate but the false positive rate was lower for ^{57}Co-bleomycin (48). One advantage of ^{57}Co-bleomycin was that it had no affinity to bone. Thus it was relatively easy to find areas of increased activity adjacent to skeletal masses (48). In a preliminary clinical trial ^{57}Co-bleomycin was found to successfully identify the presence and extent of pancreatic carcinomas (49).

In a more extensive study, ^{57}Co-bleomycin was subjected to a comparative clinical evaluation with ^{67}Ga-citrate (50). The conclusions reached, using only 50 patients, was that ^{57}Co-bleomycin displayed a greater detection rate for epidermoid carcinomas. There was found to be no difference between the ^{57}Co-bleomycin and ^{67}Ga-citrate for detection rates in adenocarcinomas (50).

b. In-111-Bleomycin

In an evaluation of ^{111}In-bleomycin as a tumor imaging agent, whole body scans were performed on a large number of patients with a variety of active tumors (50). In this study there was an 89% overall true-positive rate and an 11% false-negative rate. The true-positive rates by tumor type were: adenocarcinoma of gastrointestinal tract origin (95%), lymphoma (88%), melanoma (87%), sarcoma (82%), lung (77%), breast (77%), childhood tumors (71%), gynecologic tumors (70%) and genitourinary tumors (68%). Soft tissue and lymphatic sites of tumor, both above and below the diaphram, were easily visualized whereas hepatic and bone marrow sites of involvement were less easily discerned (50). False-positive uptake was noted in the lungs (6%), Gut (3%), mediastinum (2%) and normal breast tissue (0.8%) and in the occasional inflammatory lesion. It was concluded that ^{111}In-bleomycin was effective as a tumor-imaging agent in patients with a variety of solid tumors. Significant advantages over other similar type agents include: a broad spectrum of tumors taking up the radiopharmaceutical and a better delineation of abdominal and pelvic involvement due to lack of interference from gut uptake (50).

In another study, ^{111}In-bleomycin was evaluated as a diagnostic agent for tumors of the thorax and abdomen (51). The principal sites of bleomycin destruction was found to be the liver and spleen (5) and these were the sites where ^{111}In was found to be deposited. Excretion was found to be more exclusively by the renal route although some hepatic excretion was observed in rare instances (51). The high uptake in marrow, spleen and liver precluded the identification of lesions in or overlying these tissues. This study also concluded that ^{111}In-bleomycin accumulated in a wide variety of neoplasms. Among the tumors detected were primary and secondary carcinomas of the breast, bronchus, colon, rectum, ovary and prostate (51). Some, but not all inflammatory lesions were found to take up the ^{111}In-bleomycin (51).

Indium-111-bleomycin was evaluated as an agent for the detection of malignant melanomas (52). An overall detection rate of 64% was demonstrated in cases with known melanomas (52). The authors conclude, on the basis of a sample size of 51, that ^{111}In-bleomycin was useful as an adjunct in the evaluation of metastatic disease in malignant melanoma (52).

Another study evaluated the role of ^{111}In-bleomycin in scintigraphy in predicting the nature (benign or malignant) of palpable breast masses (53). The sensitivity (the percentage of proven malignant tumors showing abnormal accumulation) was 71%. The use of ^{111}In-bleomycin scintigraphy appears to be a promising non-invasive technique for the evaluation of palpable breast masses (53). When ^{111}In-bleomycin breast scanning was compared to other non-invasive techniques in the detection of malignant primary tumors of the breast it was found that mammography was superior to ^{111}In-bleomycin scans when patients with known breast tumors were evaluated (54). However, when the two techniques were used as screening tests, that is, when the patients examined had non-palpable masses, the specificity of mammography fell so that in this instance the over all accuracy for ^{111}In-bleomycin was better than that of mammography (54).

Indium-111-bleomycin was evaluated as a tumor scanning agent in pediatric oncology (55). A true-positive rate of 83% was found while false-negatives were 7% and false-positives were 10%. The radiopharmaceutical was found to localize in many sites which were not suspected clinically but which subsequently were shown to have malignant involvement (55). One advantage sited in this study was the lack of significant bowel activity thus making visualization of abdominal and pelvic masses easier (55).

Finally, a study has been reported involving ^{111}In-bleomycin scanning of normal patients (56). The objective was to critically evaluate the pattern of distribution in normals. A large proportion of the patients showed relatively high uptake in bone marrow. Very high uptake in the kidneys was observed in about half of the patients studied. Liver activity was equal to or greater than renal activity in all cases (56). Spleen activity was also found to be relatively high and about equal to accumulation in the bone marrow. Cardiac and lung regions showed a variable pattern of uptake with no large or unusual areas of radioactivity (56). The abdomen showed unusual accumulation in parts of the large colon, possibly indicating hepatic excretion (56). The highest concentration of ^{111}In-bleomycin was found in the liver and kidneys while little or no accumulation was found in the abdomen and pelvis (56).

c. Technetium-99m-Bleomycin

In this study, bleomycin was labeled with ^{99m}Tc using the stannous chloride and ascorbic acid method (20). In patients, positive tumor images were obtained by scintigraphy as early as 1 hour after administration (20). In 93 cases with various malignant tumors, the tumors were detected at a rate of 80%. Technetium-99m-bleomycin scintigraphy successfully detected tumors of the thyroid, lung, face, neck, breast, extremity and digestive tract and was also useful in locating metastatic lesions and brain tumors (20). It was found that ^{67}Ga scintigraphy gave a better detection rate for patients with malignant lymphomas (20). A low rate of accumulation was observed in inflammatory lesions for ^{99m}Tc-bleomycin (20). One disadvantage with ^{99m}Tc-bleomycin was observed. The high radioactivity distribution in the kidney, urinary bladder, nasal area and circulating blood pool caused difficulty in interpreting scans in some cases (20). This high accumulation of activity may be associated with the low tumor detection rates that were observed for patients with cancer of the esophagus and abdominal and pelvic organs (20).

d. Iodinated Bleomycin

Recently, bleomycin has been covalently labeled with radioactive iodine (57). Animal studies indicate that tumor to blood and tumor to muscle ratios were optimal at 6 hours post injection. The radiopharmaceutical was found to be rapidly excreted and only 83.5% of the initial dose was found in the body at 6 hours post injection (57). Blood clearance of the iodinated bleomycin was also found to be rapid with less than 0.2% of the injected dose, per gram of blood being detectable 6 hours after injection (57).

A preliminary study with ^{123}I-bleomycin was carried out in patients with cancer (57). Increased radioactivity was present in the tumor, kidneys and bladder and to a lesser extent in the thyroid, salivary glands and gut (57). The iodinated bleomycin in patients was found to be rapidly distributed and cleared from the body, in parallel with results obtained from animal studies (57). The iodine-bleomycin bond is covalent in nature with the histidine component being the most likely site for iodine incorporation (58). The conditions for covalent reactions are different from those required for chelate bond formation. Covalent reactions tend to be relative harsh and the molecule being iodinated can be degraded or modified chemically and biologically (63)(64)(67)(68)(73)(74)(81)(83)(84)(86)(87). Meyers et al. used ICl to prepare their iodinated bleomycins and a reaction time of 2 hours was required to produce a label of

85% (58). The product was found to be stable at 23°C and deiodination was found to occur at a rate of less than 0.2% per day (58). Iodination was not found to alter the bleomycin molecule chemically or chromatographically (58). Nuclear magnetic resonance (nmr) studies and mass spectroscopy studies however were not carried out.

II. Iodine

A. Absorption, Distribution and Metabolism of Iodine

The pathways of iodine physiology are well known, although the mechanism of many of the interactions remain elusive (59). Iodide is readily absorbed throughout the length of the small intestine, except for a small portion near the midpoint. This midpoint portion of the intestine, the gastric mucosa and the salivary glands are involved in active secretion of iodide into the lumens of their respective organs. Absorbed iodide is transported in the plasma as the free ion and becomes part of the total iodide pool. The pool size is larger than suggested by plasma levels because of the propensity of several extrathyroidal tissues for iodide. Many membranes do not distinguish between chloride and iodide when iodide plasma concentrations are high. There is however, a preferential concentration of iodide in the parotids, stomach, mammaries, placenta, ovaries and thyroid at physiological iodide levels (59).

Iodide is rapidly cleared from the plasma by the thyroid and the kidneys. In an adult human 70% to 80% of the total iodine is present in the thyroid, which on a dry basis contains 8-10mg of iodine. Iodide exists in other tissues but at an extremely low level, in the order of 0.01 to 0.02 ppm in muscle and blood for example (59).

The metabolic fate of iodine is determined largely by the thyroid gland and its hormones. The mechanism by which the thyroid gland concentrates iodine has not yet been clearly established. Thyroid iodide trapping is stimulated by the thyrotropic hormone of the pituitary and is inhibited by excess iodide and organic iodine as well as such inorganic ions as thiocyanate, perchlorate and nitrate (59). Iodide trapped by the thyroid is rapidly incorporated into thyroglobulin, a protein found in the colloidal matrix of the thyroid follicles. Upon hydrolysis thyroglobulin yields various iodinated derivatives of tyrosine and thyronine. Only triiodothyronine and tetraiodothyronine are physiologically important. The detailed metabolism and function of these hormones is beyond the scope of this thesis and the reader is referred to several well documented reviews (60)(61)(62). The kidneys are the only excretory pathway of iodide besides the exocrine glands and the latter excrete only a minute portion of the total.

B. Properties, Production and Clinical Uses of Iodine and the Radioiodines

Iodine has 25 radioisotopes, of these only ^{131}I, ^{125}I, and ^{123}I have been widely used for clinical purposes. A complete discussion of the properties, production and clinical uses of iodine is beyond the scope of this thesis. Comprehensive reviews however are available (63)(64).

C. Chemical Iodinations

The labeling of peptides and proteins has been extensively studied and the methods used have included chemical, enzymatic and electrolytic procedures (65)(66)(67)(68). The methods relevant to this thesis are the iodine monochloride method of McFarlane, as modified by Reif (69) and the chloramine T method of Hunter (66).

1. Iodine Monochloride and Molecular Iodine Labeling of Peptides and Proteins

The theoretical aspects of halogenation has been reviewed by Berliner (70) and Hughes (71).

The possibility that positive halogen compounds might be the substituting agents in some electrophilic aromatic reactions has been the subject of much speculation (70)(71). The kinetic characteristics of all iodinations is the dependence of the reaction rate on the INVERSE square of the iodide ion concentration (70)(71). This empirically determined kinetic data supports two reaction mechanisms both of which involve molecular iodine as a reactive intermediate (70). However, before mechanisms of iodination can be developed, it is important to discuss the interaction of iodine with the other molecular species present in the reaction medium, since their presence can profoundly affect the outcome of any iodination experiment.

2. Iodine-Solvent Interactions

Iodine is soluble to the extent of 1.1 mMol/l in water at 20°C (70). An agent such as iodide is often added to the iodine solution to form the more soluble triiodide complex (equation 1) (71).

$I_3 \rightleftharpoons I_2 + I^-$ K= 0.0013 Equation 1

The resulting equilibrium is displaced far to the left. Iodine in aqueous solution, theoretically, can react with water molecules to form a positive halogenating species (equation 2) (71).

$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$ K= 1.2 x 10^{-11} Equation 2

The formation of the reactive species is suppressed if the reaction mixture contains an excess of carrier iodide, which is often the case, and by the formation of triiodide (equation 1) which tends to consume I_2. In basic media iodine is hydrolized rapidly to give unstable hypoiodous acid (equation 3) (71).

$I_2 + OH^- \rightleftharpoons IOH \quad I^-$ K= 30 Equation 3

The hypoiodous acid reacts to give hypoiodite which can irreversibly give stable iodate (equations 4 and 5) (71).

$HOI \rightleftharpoons H^+ + OI^-$ K= 10^{-11} Equation 4

$3OI^- \longrightarrow IO_3^- + 2I^-$ Equation 5

Reaction 5 occurs rapidly at alkaline pH, and at pH 10 the reaction becomes the limiting step for any iodine labeling procedure (71). In aqueous media, iodine monochloride (equation 6) can react with water to give the same reactive species as in equation 2 (72)(73).

$$ICl + H_2O \rightleftarrows H_2OI^+ + Cl^- \qquad \text{Equation 6}$$

But it can also react with water to give molecular iodine thus (74).

$$5ICl + 3H_2O \longrightarrow 5H^+ + 5Cl^- + H^+ + IO_3^- + 2I_2 \qquad \text{Equation 7}$$

The species H_2OI^+ seems to be the feasible iodinating agent regardless of whether it comes from the ionization and hydrolysis of molecular iodine or iodine monochloride or whether it comes from the solvation of dipolar $^+I{-}{-}I^-$ or $^+I{-}{-}Cl^-$ molecules (70)(75)(76). The rate limiting step for the overall reaction sequence could be either the attack of the H_2OI^+ on the substrate or the elimination of the proton from the substrate-iodide complex (70), as is shown in the reaction sequence 8.

$$ICl \text{ or } I_2 + H_2O \rightleftarrows H_2OI^+ + I^-$$

$$H_2OI^+ + Ar - H \rightleftarrows [Ar + HI]^+ + H_2O \qquad \text{Equation 8}$$

$$[ArHI]^+ \longrightarrow Ar + I + H^+$$

(Ar represents any aromatic nucleus)

The kinetics of iodination however, is complicated by the phenomenon of catalysis by basic buffer salts (36)(70). This leads to the contention that HOI could also be the iodinating species (77). The main feature of the iodination reaction however, is not altered by the advent of this alternative interpretation of the data. The kinetic evidence (the dependence of the reaction rate on the inverse square of the iodide concentration) is also consistent with yet another interpretation when it is combined with the observation that a large isotope effect is evident with most iodination reactions (70)(78).

It has been observed that the reaction rates of iodine with deuterated substrates proceed four times slower than reaction rates with non-deuterated substrates. This is a sufficiently large isotope effect to deduce that the breaking of the carbon-hydrogen (C-H) bond is part of the rate determining step. While this evidence is not in disagreement with a reaction involving H_2OI^+ or HOI it is consistent with a mechanism of iodination by molecular iodine or iodine monochloride, in which I_2 (or ICl) and ArH are at or near equilibrium. This alternative mechanism is illustrated in equation 9 (70).

$$\mathrm{ArH} + I_2 \text{ (or ICl)} \rightleftharpoons [\mathrm{ArHI}]^+ + I^- \qquad \underline{\text{Equation 9}}$$

$$\mathrm{ArHI}^+ \longrightarrow \mathrm{ArI} + H^+$$

In this model, the loss of proton from the iodide complex is the rate limiting step. This is more consistent with the present generally accepted mechanism of electrophilic substitution in an aromatic heterocycle (79). The reaction rate of equation 9 also exhibits an inverse dependence on square of the iodide concentration (70). Reasonably large isotope effects have been observed in the iodination of imidozole by I_2 (80). The C-H bond breaking as the rate determining step of iodination is in agreement with the role of the base in buffer catalysis as assisting the breaking of the bond.

All of the iodination reactions appear to have similar kinetic characteristics and whether or not the actual reactive species is molecular iodine or some form of iodine in the +1 state is still a contentious issue that has yet to be resolved (70)(76). However, it has been suggested that the substituting agent, I_2 or H_2OI^+, may change with the substrate and the iodide ion concentration. Solutions of I_2 in water always contain some H_2OI^+. In addition, there have been reports that observed reaction rate constants can be represented by two independent terms in the overall rate equation (81)(82). It has been suggested that the first term represents iodination by molecular iodine and the second represents the reaction of H_2OI^+ (81)(82). Thus both equation 7 and 8 can explain iodinations by molecular I_2 and ICl. It should be noted however, that in solutions of ICl, the concentration of H_2OI^+ is much greater than in solutions of I_2 because the hydrolysis constant of ICl is much larger than that of I_2 (70).

The iodine monochloride reaction is of great utility in peptide and protein labeling in spite of the inconclusive nature of its mechanism. The basic method of McFarlane (65), has been modified extensively by Reif (69). The protein or peptide to be labeled is dissolved in a borate, citrate or phosphate buffer at pH 7.8. The radioactive iodine is added to this alkaline solution and then the desired amount of ICl at pH2 is added slowly and with rapid stirring. The amount of buffer at pH 7.8 that is required depends on the desired ICl/peptide ratio. In any event the amount of buffer that is used must be sufficient to keep the final pH of the reaction mixture in the optimal range of pH 6.5-7.0 (69).

In general it has been found that the higher the ICl/peptide ratio the greater is the percentage of iodine incorporated in the peptide (69). Reif (69), found that a 92.3% label could be obtained using a 16/1 ICl/peptide ratio. It was also noted the ICl is stable in acid medium at pH2 but when this pH is suddenly raised to pH 7-8 by addition of a relatively large amount of borate buffer, as would occur in an actual labeling reaction, ICl is rapidly converted into molecular iodine. The ICl and I_2 content of such a reaction mixture falls by 50% in 10 minutes at room temperature and 40% at 0^oC (69). The loss of iodination capacity is due to escape of volatile molecular iodine into the atmosphere and by the generation of stable iodide and iodate (equation 3, 4 and 5) (69).

3. Iodine-Peptide Interactions

Specific reactions of iodine and iodine monochloride with peptides has been studied by Hung (76), and others (83)(84). The principal reactions of iodine with peptides and proteins (83) can be listed in figure 2. These processes are essentially irreversible in that their equilibria lie far to the right. The oxidative reactions with sulfhydryl occurs more rapidly than the substitution reactions. Consequently, the first iodine consumed in any labeling experiment will be equivalent to the -SH content of the solution. When steric factors permit the sulfenyl-iodide (equation 12) is coupled with other sulfhydryl group to form a disulfide bridge. Organic sulfides are oxidized to sulfoxides thus similarly consuming iodine (70). These reactions occur almost instantaneously in acidic medium where I_2 has a high oxidative potential (76).

4. Iodine-Histidine Interactions.

Histidine has been proposed as an alternative site to tyrosine for the covalent incorporation of iodine into peptides and proteins (76). Hung (76), has demonstrated the feasibility of iodinating histidine under conditions which are compatible with protein and peptide stability. The exact site for iodine incorporation into histidine has been speculated to be the carbon-2 (C-2) position on the imidazole side chain (84)(84).

Imidazole is an aromatic 5-membered heterocyclic tautomer containing 2 non-adjacent nitrogen atoms. The accepted structure can be found in any standard chemistry text such as Allinger (79), or Elderfield (85). The aromatic character of the imidazole nucleus is responsible for its properties and reactivities. Fifteen resonance structures can be written for imidazole (85). These can be divided into 4 main groups on the basis of their relative contributions to the overall stabilization of the molecule. The ionic structures of imidazole are found to contribute most heavily to the resonance energy of the molecules (85). Since a resonating molecule enters into reactions through its polarized states, the imidazole moiety should possess great inherent reactivity (85). Imidazole reacts readily with iodine in form 2,4,5 triiodoimidazole (85). This reaction is catalyzed by base. The imino nitrogen, N-1, (also referred to as the pyrrole nitrogen) is capable of undergoing substitution with iodine (85), and 2,4,5 triiodoimidazole will react with excess iodine to give 1,2,4,5 tetraiodoimidazole. Iodination of imidazole with suboptimal amounts of iodine results in the formation of a mixture of 2,4 (or 2,5) diiodoimidazole (85). It has been demonstrated that the rate of iodination in the C-2 position exceeds that

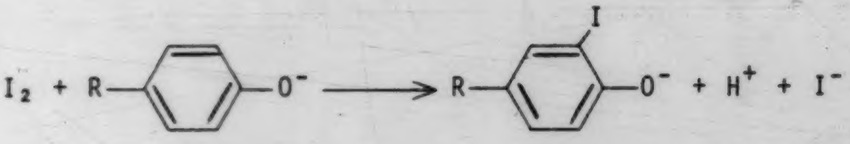
$I_2 + R\text{-}NH_2 \rightleftharpoons R\text{-}NH\text{-}I + H^+ + I^-$ equation 10

$I_2 + R\text{-}SH \longrightarrow R\text{-}S\text{-}I + H^+ + I^-$ equation 11

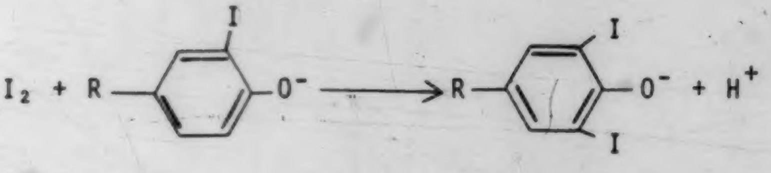
$R\text{-}S\text{-}I + R\text{-}SH \quad R\text{-}S\text{-}S\text{-}R + H^+ + I^-$ equation 12

$$\begin{tikzcd} R_2S + I_2 \arrow[r] & {[R_2SI_2]} \arrow[r, shift left] & {[R_2SI]^+I^-} \arrow[l, shift left] \arrow[dll, "H_2O"'] \\ R_2SO + 2HI & & \end{tikzcd}$$

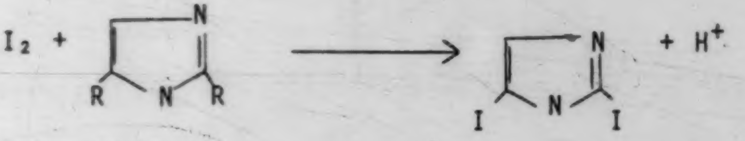
equation 13



equation 14



equation 15



equation 16

Figure 2

Principal reactions of molecular iodine with peptide functional groups (71).

Figure 2

in the C-4 or C-5 positions (85). Thus, the C-2 position was the point of preferential attack during iodination.

Two mechanisms of imidazole iodination have been proposed. The observation by Brunings (84) that iodination often required the presence of a free (unsubstituted) imino group and that the reaction was base catalyzed, let to the proposal that iodine substituted for the imino hydrogen initially, followed quickly by a base catalyzed rearrangement of the N-iodoimidazole so that iodine was finally attached to C-2 (84). This mechanism has been challenged by the fact that N-alkyl substituted imidazoles have been iodinated (76). This has led to the proposal that the iodine (either as molecular I_2 or in the I^+ state) attacks the C-2 position directly and preferentially to form an iodine containing intermediate which would slowly eliminate a proton (70)(85).

The C-iodinated imidazoles are amphoteric compounds, forming salts with metals and acids. The basic character of the imidazole system is markedly decreased by the introduction of iodine into the molecule while the acidic nature is enhanced (85). The N-iodoimidazole are generally insoluble in acid or alkali and decompose readily upon heating or by treatment with a mild reducing agent such as sulfite (85). Highly iodinated imidazoles react with aqueous sodium sulfite to form only partially iodinated products. The iodine at C-2 position has been found to be resistant to this treatment while N-iodo and other C-iodo bonds are cleaved (85).

The reaction of ICl with L-tyrosine and L-histidine, and model peptides containing these and other amino acid groups was studied by Hung (61). The peptides and amino acids were dissolved in I-125 containing borate buffers at various pH ranges and then various quantities of ICl, in anhydrous methanol, was added. It was found that when ICl is added to aqueous buffer, its rate of hydrolysis to molecular I_2 is dependent upon pH and the presence or absence of certain peptides (76). At pH 4, and 21°C iodine (I_2) was not detectable before 96 seconds and its concentration reached a maximum 4 to 5 minutes later. The half-life of ICl under these conditions was determined to be 22 minutes. When L-tyrosine was present at pH 4, the iodination reaction was complete in 12 seconds and all the ICl was consumed (76). In contrast, L-histidine catalyzed the hydrolysis of ICl as indicated by an undelayed appearance of I_2. The total amount of iodine released remained the same as the control and no reaction was observed with L-histidine for 2 minutes. Then, liberated iodine slowly disappeared with a half-life of 9 minutes indicating that a slow reaction was taking place between iodine and the protonated form of histidine (76). The catalytic influence of free histidine on the rate of ICl hydrolysis was studied in a variety of L-histidine containing peptides (76). Some peptides induced an instantaneous release of molecular I_2 from ICl, others demonstrated a very short lag period (seconds) before free molecular I_2 was detectable while other peptides demonstrated no catalytic behavior towards ICl hydrolysis (76). These findings suggested that the conformation of the peptide in aqueous solution and the degree of freedom of the L-histidine side chain, played an important role in the catalytic influence of the imidazole ring in ICl hydrolysis (76). In an aqueous buffer, at pH 7.4, free iodine was released immediately from ICl. L-tyrosine reacted in less than 10 seconds under these conditions, and no free iodine was detectable. L-histidine at pH 7.4 reacted also within 10 seconds and free iodine was not detectable. This is in sharp contrast with L-histidine at pH 4. Hung (76), also found that L-tyrosine and L-histidine were iodinated almost instantaneously at pH 9.5. These studies pointed out several pertinent facts. First, the catalytic behavior of imidazole can be profoundly influenced by the pH of the medium and by the adjacent peptides in the molecule. Secondly, the ICl may either react directly with the side chain of an appropriate peptide or it may be first hydrolyzed to molecular iodine which then reacts with the peptide group (76).

b. The Chloramine T-Method of Labeling Peptides and Proteins

The second chemical method of iodinating proteins and peptides relevant to this thesis is the Chloramine T method (66)(84). Chloramine T is the sodium salt of N-monochloro-p-toluenesulfonamide (87). It is a mild oxidizing agent and slowly liberates hypochlorous acid in aqueous solution. It has been widely used for iodination of many proteins (66). The exact mechanism of the reaction is not known. Presumably, a complex of iodine is formed with the sulfonamide in which the iodine carries a positive charge. The reaction readily goes to 100% substitution of iodine into the protein or peptide (86).

Initial experiments were performed on high molecular weight proteins using a 1 to 1 molar ratio of protein to iodide in a total reaction volume of 0.3ml (66). One molecule of chloramine T was required for every one of iodide under these conditions of high protein concentration and high iodide to protein molar ratios. At lower concentrations of iodide relatively more chloramine T was required and for trace labeling procedures at least 100 molecules of chloramine T may be required for every molecule of iodide (86). The optimal pH for the iodination of proteins by the chloramine T method was pH 7.5 (86).

The general procedure for iodination of peptide or protein consisted of adding first the protein (peptide) and then chloramine-T to a buffered solution of sodium iodide-125 (66)(86). The reaction was stopped by the addition of sodium metabisulfite. The reducing agent was added immediately after the addition of chloramine-T. The reducing agent should not be added in excess since it can modify the protein being iodinated (86). The volume of the reaction mixture was minimized because the yield of the reaction was found to be dependent upon the concentration of the protein (86)(88). The labeling yield was also influenced by the nature of the specific protein being iodinated (86). McConahey and Dixon (89), have modified the protein iodination procedure of Hunter and Greenwood. Their method provides for easy and efficient iodination of microgram and milligram quantities of proteins and peptides. The modified procedure provides for low chloramine-T concentrations and long reaction times. The advantage claimed for this modified technique has been less protein denaturation and consequently an improvement in the quality of iodinated protein available for in vivo studies. The chloramine-T to protein ratios used in this study were 1/1000, those employed originally by Hunter and Greenwood. Reaction times were up to 5 minutes and labeling efficiencies varied from 40% to 90% depending on the number of readily available reducing groups in the specific protein being labeled (89).

EXPERIMENTAL METHODS AND MATERIALS

I. Materials

A. Animals

Young male adult Swiss mice, 20-25 grams, were used for tissue distribution and excretion studies. The mice were used 7-10 days after receipt and were allowed food and water *ad libitum*.

Tumor bearing mice were prepared as follows: Mice, with Ehrlich's ascites tumor cells cultured in their abdominal fluid, were killed and the cells aspirated and diluted with normal saline (1:1). The number of cells in the suspension was determined with a Coulter counter and the cells diluted again with normal saline until approximately 2 million cells were contained in 0.1ml. Then, 0.1ml of the diluted cell suspension was injected subcutaneously into the right femoral region of each mouse. Mice were kept 6 to a cage and 50mg of ampicillin was dissolved in their drinking water to prevent bacterial infection. Tumors were allowed to grow 7 days.

B. Chemicals, Solutions and Materials

All chemicals, reagents and solvents were of Reagent or A.C.S. grade.

1. Bleomycin Sulfate

Bleomycin sulfate for parenteral use was obtained from Bristol Laboratories, Candiac, P.Q. Bleomycin sulfate is a mixture of bleomycin A_1, A_2, demethyl A_2 and B_2 principally. The A_2 and B_2 fractions can comprise from 75% to 97% of the mixture by weight (7).

2. Bleomycin A_2 Working Standard

Bleomycin A_2 working standard was obtained as batch number BWS-8 from the National Institute of Health of Japan, Tokyo, Japan.

3. Iodine-125

Sodium iodide-125 ($Na^{125}I$), iodination grade, was obtained carrier free in 0.1 N NaOH from International Chemical and Nuclear Corporation of Montreal. Radioactive concentration of the solution was 396 mCi/ml.

4. Indium-114m

Indium-114m-chloride ($^{114m}InCl_3$) was obtained carrier free in 0.1 N HCl from New England Nuclear, Dorval, P.Q.

5. Ion Exchange Resins

a. Dowex 1x4

Dowex 1x4 anion exchange resin, Bio Rad Laboratories, Richmond, California. The resin is composed of polystyrene with quaternary ammonium functional groups in the chloride form. A large pore and a mesh of 100-200 was used. The resin was allowed to hydrate for 24 hours in 0.2 N HCl and then washed to neutrality with double distilled water. The resin was kept moist until used. Dowex minicolumns, for purifying iodinated bleomycin reaction mixtures, were made by firmly layering the resin between frittered glass discs in a standard 25ml syringe. Layers of Dowex 1-2mm thick were used to purify trace labeled bleomycin reaction mixtures while 0.5-1.0cm layers were used to purify reaction mixtures containing mCi quantities of iodine

b. Amberlite IRC-50

Amberlite IRC-50 is a weakly acidic cation exchange of the carboxylic acid type in the hydrogen form and was supplied by Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A. A mesh size of

16-50 was used. The resin was hydrated with double distilled water for 24 hours before use.

6. Chloramine-T MW 281.70

Chloramine-T is the sodium salt of N-monochlo-p-toluenesulfonamide. Laboratory reagent grade was obtained from British Drug House of Poole, England. Chloramine-T solutions of 2, 4, 15 and 40 mg/ml were prepared by dissolving the required amounts in 100 ml of double distilled water.

7. Iodine Monochloride MW 162.37

Iodine monochloride reagent grade was obtained from Matheson, Coleman and Bell, Norwood, Ohio. Upon receipt of the reagent 15% V/V concentrated HCl was added to prevent decomposition of the iodine monochloride. Iodine monochloride, 3.6mM for iodination was prepared by diluting the stock solution 1:6250 with 95% ethanol. The solution was freshly prepared and stored in a freezer until immediately before use. The stock solution of iodine monochloride was standardized by titrating an aliquot of a 1:250 dilution in 95% ethanol with a standardized 0.05 N sodium thiosulfate solution. The endpoint occurred when the characteristic yellow color of ICl faded (72). The equation for the reaction is:

$$2\ Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2NaI$$

8. Sephadex G-10

Sephadex G-10 for gel filtration chromatography was obtained from Pharmacia Fine Chemicals of Uppsala, Sweden. A particle size of 40-120 microns was used. Before use, the gel was hydrated for

24 hours in double distilled water. The Sephadex slurry was then poured into 25cc syringes. A plug of glass wool was used to retain the Sephadex in the syringe barrel. Void volumes for each column was obtained by the use of high molecular weight calibration dyes.

9. Sodium Thiosulfate MW 248.18

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) was obtained as crystals from B.D.H. Pharmaceuticals, Toronto. A 3.6 mM solution was prepared by dissolving 0.149g of $Na_2S_2O_3 \cdot 5H_2O$ into 100C ml of double distilled water. The solution was made fresh and discarded after 24 hours.

10. Sodium Metabisulfite MW 190.13

Sodium metabisulfite ($Na_2S_2O_5$) anhydrous was obtained from B.D.H. Pharmaceuticals, Toronto. The solution was prepared by dissolving 4.0g of $Na_2S_2O_5$ into 100.0 ml of double distilled water. The solution was prepared fresh and discarded after 24 hours.

11. Phosphate Buffer

Phosphate buffer 0.5 M, was prepared from 0.5 M solutions of monopotassium phosphate (KH_2PO_4) anhydrous and disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), both obtained from Fisher Scientific Company, Fair Lawn, New Jersey. The proportions of each solution needed to produce the buffer at the desired pH was determined from suitable tables (90).

12. Alkaline Borate Buffer

The alkaline borate buffer at pH 7.8 was prepared by dissolving 18.7 gm of sodium chloride (NaCl) and 24.74 gm of boric acid (H_3BO_3) in 90 ml of 1.0 N NaOH, then the volume was made up to 1000 ml with double distilled water (90).

13. Other Chemicals and Reagents

a. NaOH, 0.1N, standard volumetric solutions of Anachemia Chemicals Ltd., Montreal, P.Q.

b. NaOH, 1.0N, standard volumetric solutions of Anachemia Chemicals Ltd., Montreal, P.Q.

c. HCl, 1.0N, standard volumetric solutions of Anachemia Chemicals Ltd., Montreal, P.Q.

d. Sodium Thiosulfate Standard 0.05 N, standard volumetric solutions of Anachemia Chemicals Ltd., Montreal, P.Q.

e. Ammonium Acetate ($Nh400CCH_3$), Fisher Scientific Company, Fair Lawn, N.J., U.S.A.

f. Ammonium Chloride (NH_4Cl) Fisher Scientific Company, N.J., U.S.A.

g. Boric Acid (H_3BO_3) Fisher Scientific Company, Fair Lawn, N.J., U.S.A.

h. Cupric Sulfate ($CuSO_4$) McArthur Chemical Company, Montreal, P.Q.

i. Methanol (CH_3OH) McArthur Chemical Company, Montreal, P.Q.

j. Ethanol (C_2H_5OH) McArthur Chemical Company, Montreal, P.Q.

k. Chloroform ($ChCl_3$) McArthur Chemical Company, Montreal, P.Q.

14. Eastman Chromatogram Sheets

Eastman Chromatogram sheets #6061 are mylar backed silica gel 20x20 cm sheets obtained from Eastman Kodak, Rochester, N.Y.

15. Gelman Chromatography Media

Gelman Chromatography Media I.T.L.C. type S.G.[a] was obtained in 5x20 cm sheets from Gelman Instrument Company, Ann Arbor, Michigan.

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[a] Trademark (Gelman Instruments, Ann Arbor, Mich.). For instant thin layer chromatography silica gel type.

16. Kieselgel, Camag - For Thin Layer Chromatography (tlc)

Silica gel for thin layer chromatography was obtained from Fraser Medical Supplies Ltd., Vancouver, B.C. The silica gel, 100g, was slurried with 120 ml of double distilled water. Calcium sulfate, 2%, was used as binder. A chromatogram spreader from Quickfit Instrumentation Company was used to make glass 5x20 cm and 20x20 cm plates containing 0.5 mm layers of silica gel. The layers were allowed to air dry at least 24 hours before use.

17. Siliconizing Fluid

A stock silicone fluid was obtained from Arthur H. Thomas Company, Philadelphia, Pa. A dilute siliconizing fluid was prepared by mixing 5.0 ml of stock silicone fluid with 95.0 ml of carbon tetrachloride. The glassware, previously soaked in a chromic bath for 24 hours, rinsed with water and oven dried, was immersed in the prepared siliconizing fluid. The vessels were air dried for 20 minutes then oven dried for 2 hours at 100°C.

II. Experimental Methods

A. Chromatographic Properties of Bleomycin Complex, Bleomycin A_2, Iodinated Bleomycins, ^{114m}In-bleomycin A_2, $Na^{125}I$ and $^{114m}InCl_3$

1. Characterization on Silica Gel TLC

The contents of bleomycin complex vial was weighed and then dissolved in 5.0 ml of water. Five mg of bleomycin A_2 working standard was dissolved in 5.0 ml of water. Sodium iodide-125 and $^{114m}InCl_3$ were diluted in water so that 0.05 uCi was contained in 0.1 ul of solution. The pH of the $InCl_3$ was adjusted to 7.0

before chromatographic analysis. Ten microliters of iodinated bleomycins that had been purified on Dowex columns and 10 ul aliquots of ^{114m}In-bleomycin A_2 taken directly from the reaction mixture, were diluted to 1.0 ml with water. Then 20 ul of each dilution was spotted on silica gel tlc plates, prepared as previously described. The chromatograms were developed 10 cm in 10% NH_4OOCCH_3:CH_3OH(1:1) at 20°C. The chromatograms required about 2.5 hours to develop. Plates were allowed to air dry briefly. The chromatograms containing non-radioactive bleomycin complex and bleomycin A_2 working standard were visualised under short wave ultra violet (uv) light. The chromatograms containing the radioactive substances were divided into 1 cm segments beginning at a base line 1/2 cm below the origin. The segments were scrapped into polyethylene counting vials and counted for 1 minute in an appropriately calibrated gamma counter (Beckman Biogamma Counting System #167776, Beckman Instruments Inc., Fullerton, Calif.).

2. Characterization on Eastman Silica Gel #6061

Ten microliters of each of the bleomycin solutions, the iodinated bleomycins and the Na^{125}I, prepared as previously described, were spotted on 1x20 cm strips of Eastman #6061 and developed 13-15 cm at 20°C with 95% ethanol in 2.5x20 cm test tubes that were sealed with corks. In-114m-chloride was adjusted to pH 6.5-7.0 before chromatographic analysis, then 10 ul aliquots of the $^{114m}InCl_3$ and ^{114m}In-bleomycin A_2 were spotted on Eastman #6061 and developed as before in 10% NH_4OOCCH_3:CH_3OH(1:1). The chromatograms required about 2-2.5 hours to develop 13 cm. The strips containing the unlabeled bleomycins were visualized by uv light, as before. The chromatogram containing the radioactive substances were cut into 1 cm segments beginning 1/2 cm below the origin. Each segment was analyzed for radioactivity, as before.

3. Characterization on Gelman ITLC Media SG Type

Ten microliters of each of the solutions, prepared as previously described, were spotted on 5x10 cm sheets of Gelman chromatography media. The chromatograms were developed 7.0 cm in Seprachrom[a] minitanks using 95% ethanol as the mobile phase. The chromatograms required 7 minutes to develop. The strips were dried and visualized under uv light for locating the unlabeled bleomycins, while for the radioactive compounds, the strips were divided in two and each half was assayed by a gamma counter as before.

4. Characterization on Sephadex G-10 Minicolumns.

One hundred microliters of each of the solutions containing the bleomycins, iodinated bleomycins and Na^{125}I were placed on separate Sephadex G-10 minicolumns prepared as previously described The minicolumns were then eluted with water at a rate of 0.2 ml/minute. The eluates containing the bleomycins were monitored in a uv spectrophotometer set at 254 nm (Beckman DB Spectrophotometer, Beckman Instruments Inc., Fullerton, Calif.).

The eluates were carried from the column to standard 1 cm flow cells by fine bore cannula tubing. The eluates containing

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[a] Trademark of Gelman Instrument Company, Ann Arbor, Mich.

^{125}I were monitored as they passed through fine bore cannula tubing in front of an appropriately shielded 3"x3" NaI(Tl) crystal detector system. The detector system consisted of a Canberra single channel analyzer (sca) unit, model 1437 and a 10" Beckman recorder. An Ortec high voltage power supply model 456 completed the system.

B. Isolation and Purification of Bleomycin A_2

The contents of 1 vial of bleomycin complex (Blenoxane-Bristol Laboratories), was weighed and the 8.2 mg was dissolved in 0.5 ml of water. The solution was streaked across the origin of a 20x20 cm silica gel tlc Plate prepared as described previously. The chromatogram was developed as previously described, dried and visualized under uv light. The bleomycin A_2 component appeared as a heavy streak centered at Rf 0.35. The band was scrapped from the plate, packed over a plug of glass wool in a 5 cc syringe and washed with 5.0 ml of cold acetone. The eluate was discarded. The syringe was connected to the flow cells in a spectrophotometer, as described previously and then eluted with methanol, acidified to pH 4.6 with 1.0 N HCl. The flow rate was adjusted to 0.2 ml per minute and the eluate was collected when the absorbance increased from 0.0 and collected until the absorbence returned to 0. The eluate was evaporated to dryness and then taken up in 1.0 ml of water. The yield was determined by measuring the absorbance of a 1:25 dilution of the isolated bleomycin A_2 fraction at 254 nm in a spectrophotometer. A standard curve using bleomycin A_2 working standard was prepared and is found in Appendix 1.

C. Quality Control of Radionuclides

1. Radionuclidic Purity

a. Indium-114m

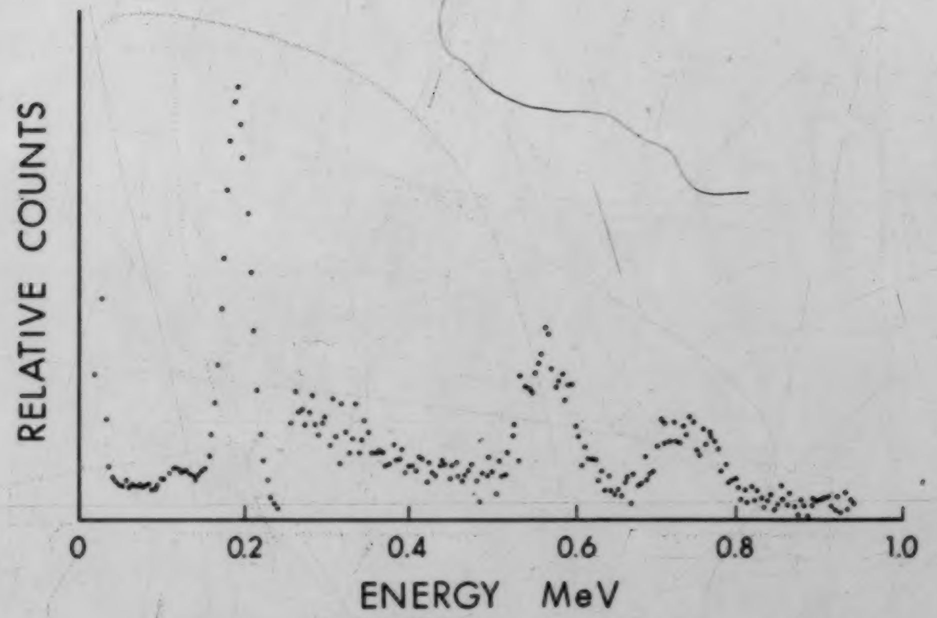
Indium-114m was obtained as $InCl_3$ in 0.1N HCl as previously described. The specific activity of the stock solution was 51.4 mCi/g. The radionuclide purity of the $^{114m}InCl_3$ was confirmed by its energy spectrum. A sample of the stock solution was placed in front of a NaI(Tl) detector (Picker Autowell II Picker Corp., Cleveland, Ohio). The energy spectrum was stored in a multichannel analyzer (Northern Scientific, Middleton, Wisc.) and then plotted on an X-Y plotter. The resulting spectrum is shown in figure 3. Peaks were seen at 0.19 MeV, 0.56 MeV and 0.72 MeV. These agree with published gamma emissions of ^{114m}In (91). As no other peaks were evident, the radionuclidic purity of the $^{114m}InCl_3$ stock solution was acceptable.

b. Iodine-125 ($Na^{125}I$)

The ^{125}I was obtained as previously described. The concentration of the stock solution was 396 mCi/ml. The radionuclidic purity of the stock solution was confirmed by its energy spectrum shown in figure 4 and was in agreement with the published gamma emissions of ^{125}I (91). As no other peaks were evident, the radionuclidic purity of the $Na^{125}I$ solution was accepted.

D. Preparation of ^{125}I-Bleomycin Complex, ^{125}I-Bleomycin-A_2 and ^{114m}In-Bleomycin A_2

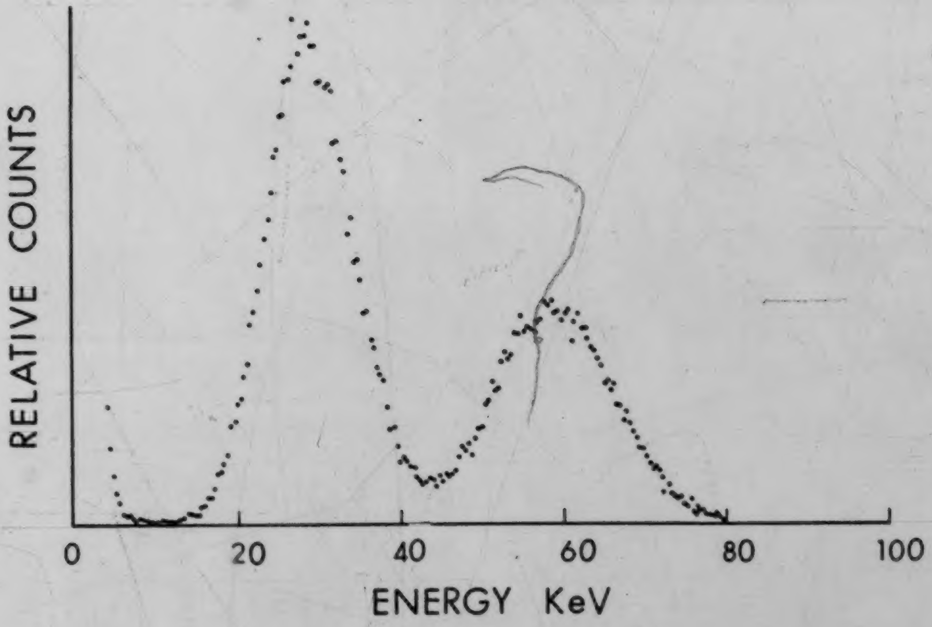
All glassware was soaked in chromic acid for 24 hours and



^{114m}INDIUM SPECTRUM[a] OBTAINED WITH A SODIUM IODIDE SCINTILLATION DETECTOR

[a] (with 1/16" glass beta filter)

Figure 3



^{125}I ENERGY SPECTRUM OBTAINED WITH A SODIUM IODIDE SCINTILLATION DETECTOR

Y full scale 4 x 2^8 counts in 64 secs.

X full scale 100 KeV.

Figure 4

then rinsed with freshly boiled distilled water before use. Glassware used for reaction vessels and for the manipulation of reaction mixtures were siliconized, as previously described.

The contents of a bleomycin vial was dissolved in 1.0 ml of water to produce a stock solution of 8.2 mg/ml. Four separate reaction mixtures were prepared as follows:

Bleomycin complex 8.2 mg/ml	20 ul
Na^{125}I 1 uCi/ul	20 ul
Phosphate buffer 0.5M, pH 7.8	20 ul.

Then reactions were carried out using 20 ul of each of the chloramine-T solutions prepared at 2, 4, 15 and 40 mg/ml. These solutions gave chloramine-T concentrations of 40, 80, 300 and 800 ug in each reaction mixture. The reaction time was 120 seconds at 20^{o}C. Reactions were terminated by the addition of 20 ul of 40 mg/ml sodium metabisulfite solution.

a. Blank Reactions

A blank reaction was performed in the manner described above except that the bleomycin was deleted from the reaction mixture and replaced by an equal volume of buffer.

b. Zero Time Reaction

A zero time reaction was performed in which the chloramine-T was deleted from the reaction mixture and replaced by an equal volume of buffer.

All reaction mixtures were monitored by gel filtration chromatography using Sephadex G-10 minicolumns. The eluates were monitored by a shielded 3"x3" NaI(Tl) crystal detector system, as previously described. A 2.0 ml fraction was collected starting at the void volume. This fraction contained the iodinated bleomycins. Free ^{125}I was collected in a 6-8 ml fraction beginning at twice the void volume of the column.

The chromatographic properties of the G-10 eluates from the iodinated bleomycin, blank and zero time reaction mixtures were assessed on silica gel tlc and on Eastman #6061 silica gel strips. The mobile phases were 10% NH_4OOCCH_3:CH_3OH(1:1) and 95% ethanol respectively. Reactions were done in triplicate and all chromatograms were done in duplicate.

c. Determination of Optimal Reaction Times

The chloramine-T iodination reaction was tested at time intervals of 30, 60, 120, 180 and 300 seconds. Five reaction mixtures were prepared as previously described and to each was added 20 ul of 40 mg/ml chloramine-T solution. The reactions were terminated at the designated intervals by the addition of 20 ul of sodium metabisulfite solution. The eluates were subjected to gel filtration chromatography and assayed on silica gen tlc and Eastman #6061 strips as previously described.

d. Determination of Optimal Reaction pH

The efficiency of the chloramine-T reaction was assessed at pH values of 6.0, 7.0, 7.5, 8.5 and 9.0. The pH values of 6-7.5 were supplied by phosphate buffers while the pH values of 8.5 and 9.0 were supplied by 0.25M alkaline borate buffers. The buffer solutions at the desired pH value were made according to tables found in Documenta Geigy (90). The pH of each buffer was verified on a single electrode pH meter (Beckman Zeromatic II, Beckman Instruments Inc., Fullerton, Calif.). Any necessary pH adjustment was accomplished using 0.1N HCl or 0.1N NaOH.

Five reaction mixtures were prepared, each one with a different buffer pH and to each mixture was added 20 ul of 40 mg/ml chloramine-T solution. The reaction was allowed to proceed at 20°C for 60 seconds before termination with sodium metabisulfite solution. Reaction mixtures were subjected to gel filtration chromatography and the eluates were assayed as before.

e. The Determination of Optimal Reaction Temperatures

Two identical reactions, using quantities as previously described, were carried out, one at 20°C and one at 4°C. Reactions were terminated after 60 seconds. The reaction mixtures were processed on sephadex G-10 columns and the eluates were assayed as before.

2. Preparation of ^{125}I-Bleomycin Complex and ^{125}I-Bleomycin A_2 Using the Iodine Monochloride Method

The ICl stock solution was diluted with 95% ethanol, to give a 3.6 mM solution which was then used for iodination procedures. The bleomycin was dissolved in alkaline borate buffer at pH 7.8 (91), to give a concentration of 1 mg/ml. Since control of pH was found to be important in other similar studies (58)(74), various quantities of 3.6 mM ICl solution were added to 15 ml of borate buffer and the pH was determined using pH Universal Indicator Sticks (E. M. Laboratories, Elmsford, N.Y.). It was found that up to 3.0 ml of

ICl solution could be added to the buffer system without any appreciable change in pH. Quantities of 3.5-4.0 ml of ICl produced changes of 1 pH unit in the buffer system. Thus 0.3 ml of ICl could be added to 1.5 ml of buffer without affecting the pH of the buffer system.

The iodination procedure that was developed was a modification of the one described by Reif (69). Into a siliconized reaction vessel was placed 1.5 ml of the bleomycin in borate buffer solution at pH 7.8. To this was added the desired amount of ^{125}I. For trace iodinations 20 uCi was added while for higher specific activity iodinations, 2.5 mCi or more was used. The solution was constantly stirred using a small polyethylene coated magnetic stirring bar. The ICl was added in three 0.1ml aliquots at 10 minute intervals. The reaction was allowed to proceed for 30 minutes at 20°C before it was terminated with 0.3 ml of 3.6 mM sodium thiosulfate solution.

The reaction mixtures were not subjected to gel filtration chromatography but were applied directly to 1x20 cm strips of Eastman #6061 silica gel and 5x10 cm sheets of Gelman ITLC. The chromatograms were developed using 95% ethanol as mobile phase and assayed as previously described. The balance of the reaction mixture was allowed to percolate through a small Dowex 1x4 column, prepared as previously described, to remove unreacted iodide. The columns were washed with 0.1 ml of alkaline borate buffer to remove as much of the ^{125}I-bleomycin as possible that was trapped in the gel bed. The chromatographic behavior of the ^{125}I-bleomycin was determined using silica gel tlc plates and assays for reaction yield and radiochemical purity was performed on Eastman #6061 and Gelman ITLC, as previously described.

a. The Effect of ICl Concentration on Reaction Yield

Five reaction mixtures were prepared, as follows, in siliconized reaction vessels containing magnetic stirring bars.

Bleomycin complex 1 mg/ml in borate buffer at pH 7.8	1500 ul
$Na^{125}I$ in water 1 ug/ul	20 ul

Duplicate 5ul aliquots were removed from each reaction vessel for assay on Eastman #6061 and Gelman ITLC at zero time. Then, 100 ul of 3.6 mM ICl was added to 3 of the reaction vessels, 300 ul was added to a fourth and no ICl was added to the fifth vessel. At 3 and 10 minute intervals duplicate 5ul aliquots were again removed for assay. Immediately after the 10 minute aliquots were removed for assay, 100 ul of the 3.6 mM ICl solution was added to only 2 of the reaction vessels. The reactions were allowed to proceed for a further 10 minutes (t=20 minutes) before all reaction mixtures were again sampled and assayed as previously described. Then at t=20.5 minutes, 100 ul of the ICl solution was added to only 1 reaction vessel. Thus, at t-20.5 minutes there was 1 reaction vessel which contained no ICl, one which contained 100 ul of ICl solution, one reaction mixture which contained 200 ul of ICl solution, one reaction mixture which contained 300 ul of ICl added all at once and one which contained 300 ul of ICl added in three 100 ul aliquots at 10 minute intervals. The reactions were allowed to proceed a total of 180 minutes before they were terminated with 300 ul of 3.6 mM sodium thiosulfate solution.

Aliquots for assay were also removed at 30, 60, 90 and 180 minute intervals. A blank reaction mixture was processed in parallel with this group of reactions. The blank reaction mixture contained all of the ingredients except the bleomycin.

In a second series of reactions, the stock bleomycin solution of 1 mg/ml in borate buffer was used to prepare 3 reaction mixtures containing 150 ug, 500 ug and 1500 ug of bleomycin complex in a reaction volume of 1.5 ml. To each of these reaction mixtures was added 20 uCi of $Na^{125}I$, followed by 100 ul of 3.6 mM ICl solution. Further 100 ul aliquots of ICl were added at 10 minute intervals until a total volume of 300 ul had been added to each reaction vessel. Duplicate 5 ul aliquots for assay were removed from each reaction mixture at 10 minute intervals immediately before the aliquots of ICl were added. Assays were performed on Eastman #6061 and Gelman ITLC as previously described. All reactions were carried out at 20°C.

b. The Effect of Temperature on Reaction Yield

Two identical iodination reactions were carried out as previously described. One reaction mixture was carried out at 20°C while the second reaction was carried out at 4°C. The reactions were terminated after 20 minutes and assayed in triplicate on Eastman #6061 and Gelman ITLC.

3. Preparation of ^{125}I-Bleomycin A_2

Bleomycin A_2, isolated from the complex as previously described and bleomycin A_2 working standard were iodinated by adding

3 successive 100 ul aliquots of ICl at 10 minute intervals. Reactions were carried out at 20°C. and were terminated after 30 minutes.

4. Preparation of Iodinated Bleomycin for Nuclear Magnetic Resonance (nmr) Studies

One of the structural components of the bleomycin molecule is B-hydroxyhistidine (22)(23). B-hydroxyhistidine contains an imidazole ring side chain as its principal functional group. The most likely site of covalent iodine incorporation into bleomycin is at the C-2 position on the imidazole ring (40)(41)(58)(76). The proton at the C-2 position produces a doublet signal at 9.16δ (with coupling constant $J=1.5$ cps), when the molecule is analyzed by nmr spectroscopy (23). The doublet signal from the proton at C-2 should diminish in size as the proton is replaced by iodine and the signal should completely disappear from the nmr spectrum when the electrophilic substitution by iodine at the C-2 position is at or near completion. This would establish the position where iodine is incorporated into the bleomycin molecule.

The following reaction mixture was prepared,

Bleomycin A_2 1 mg/ml in borate buffer at pH 7.8	1500 ul
$Na^{125}I$ 1 uCi/ul	20 ul
$Na^{127}I$ 1g/ml	150 ul

To this solution was added 300 ul of ICl in three 100 ul aliquots at 10 minute intervals. The reaction mixture was monitored every 20 minutes until 80% of the ^{125}I present in the reaction mixture was found to be at the origin on the Gelman chromatograms. This required a 3-4 hour reaction time at 20°C. The non-radioactive iodide, $Na^{127}I$, was added in sufficient quantity to give an iodide: peptide molar ratio of 1:1. The trace quantity of ^{125}I was added to the reaction mixture only to monitor the progress of the iodine incorporation. When about 75% of the radioactivity appeared at the origin of the chromatograms the reaction was terminated with 300 ul of 3.6 mM. sodium thiosulfate solution. The reaction mixture was diluted to 20 ml and about 1.5 g of amberlite IRC-50 cation resin was added. The reaction mixture and the resin were stirred for 2 hours. The resin was separated from the supernatant, dried and then packed into a small glass column. The resin was eluted with methanol acidified to pH 4.6 and the eluate was monitored by a shielded 3"x3" NaI (Tl) crystal and an sca as previously described. The eluate was neutralized with 0.1N NaOH and evaporated to dryness. The residue was washed with 5.0 ml of cold acetone and then dried again. The residue was then taken up in deuterated methanol (CD_3OD), and scanned by a 100 mHz analytical NMR spectrometer (Model A, 100 D, Varian Associates, Bellvue, Wash.). A $10^{-3}M$ solution of unlabeled bleomycin in CD_3oD was scanned as a reference spectrum. The spectra was scanned from delta 7.90 to 9.25.

The nmr scans were performed 100 times with noise signals being subtracted from each scan. In this way the desired doublet at 9.16δ could be visualized.

5. Preparation of ^{114m}In-Bleomycin A_2 For Tissue Distribution Studies

In-114m bleomycin A_2 was prepared after the method of Grove et al. (9). Two mCi of $^{114m}InCl_3$ was diluted to 3.0 ml with 0.1N HCl then 5 mg of bleomycin A_2 was dissolved in the solution. The pH of the solution was adjusted to 6.5-7.0 with 0.05 N and 0.01 N NaOH. The solution was chromatographed on silica gel tlc and assayed on Eastman #6061. In both cases the mobile phase was 10% NH_4OOCCH_3: CH_3OH (1:1).

E. Stability of Iodinated Bleomycin A_2 Solutions

1. Stability of Iodinated Bleomycin A_2 Solutions at 4°C. and 20°C.

Solutions of iodinated bleomycin A_2 were purified on Dowex 1x4 columns and then stored in stoppered clear glass vials at 20°C. and 4°C. for 30 days. Triple 10 ul aliquots were removed and assayed on Eastman #6061.

This was done daily for a period of 10 days, thereafter aliquots were removed and assayed on day 20 and day 30. Silica gel tlc chromatography was performed on days 1, 10, 20 and 30.

2. Stability of Iodinated Bleomycin A_2 to Elevated Temperatures

Solutions of iodinated bleomycin were assayed on Eastman #6061 and chromatographed on silica gel tlc. The solutions were placed in unstoppered glass vials and then immersed into a boiling water bath for 60 minutes. The solutions were cooled and made up to volume with buffer. The iodinated bleomycin solutions were then again assayed on Eastman #6061 (95% ethanol as solvent) and chromatographed on silica gel tlc with NH_4OOCCH_3:CH_3OH (1:1) as solvent.

3. Stability of ^{125}I-Bleomycin A_2 After Incubation with Cu^{+2}

Bleomycin has been found to preferentially form stable chelates with copper (1). The preferential nature of the copper-bleomycin association can be demonstrated by the fact that ^{111}In-bleomycin is completely destroyed in vitro by the presence of Cu^{+2} (10)(11). The presence of Cu^{+2} would completely destroy an iodine-bleomycin adduct if the iodine were merely chelating since the Cu^{+2} would compete with iodine for chelation sites on the ligand. Therefore, if iodinated bleomycin is stable in the presence of Cu^{+2}, a covalent association rather than chelate information would be indicated. To test this, ^{125}I-bleomycin A_2 was incubated with an excess of Cu^{+2}.

Solutions of ^{125}I-bleomycin A_2 were chromatographed on silica gel tlc as previously described and assayed on Eastman #6061. A copper chloride ($CuCl_2$) solution was prepared so as to give 10 ug Cu^{+2}/ul. Then 0.1 ml of the Cu^{+2} solution was incubated with 0.1 ml of the ^{125}I-bleomycin A_2 solution for 30 minutes at 20°C.

Triplicate 10 ul aliquots of the copper-^{125}I-bleomycin A_2 solution were chromatographed on silica gel tlc and assayed on Eastman #6061 strips as previously described.

F. Tissue Distribution Studies

The tissue distribution of ^{125}I bleomycin complex and ^{125}I-bleomycin A_2 were studied in normal mice and mice bearing Ehrlich's ascites tumor in solid form and then compared with the tissue distribution of ^{114m}In-bleomycin A_2 in similar tumor bearing mice. Tumors were allowed to grow for 7 days in the right femoral region before tissue studies were undertaken.

A preliminary study was completed on a small number of normal and tumor-bearing mice in which a wide variety of tissues were excised and assayed for radioactivity. This study indicated the tissues of interest and also the time intervals at which the tissue uptake of radioactivity would be meaningful.

The radioactive solution, containing about 27 uCi in 0.1 ml, was injected slowly via the tail vein. At specified time intervals after injection the mice were sacrificed by cervical dislocation. A 0.1 ml sample of blood was immediately obtained from the heart. The blood was transferred to a glass counting vial. The entire liver, kidney, spleen and testes were excised and aliquots of muscle and lung were taken. When applicable 2 aliquots of tumor were excised. The tissues and organs were blotted free of blood, weighed and transferred to glass counting vials. The radioactivity in the organs and tissues were assayed for 1 minute in an appropriately calibrated gamma counter (Nuclear Chicago 1185, Searle Analytic Inc., Des Plaines, Ill.).

In another preliminary study excised organs and tissues were divided into 3 portions. One portion was counted intact while the other two portions were solubilized in 4M KOH and concentrated HNO_3. However, it was found that counts/mg obtained for intact tissues were about the same as counts/mg obtained for solubilized tissues. It was therefore decided to assay for radioactivity only intact organs and tissues. In all cases the radioactivity in the carcass was also determined. The carcass was placed in an open top plastic bottle 12.5 cm long and 5.5 cm in diameter which was then lowered into a small animal whole body counter. The radioactivity was detected by two 3"x3" NaI (Tl) crystals and recorded.

Mice were sacrificed at intervals of 1, 2, 3, 4, 6, 12 and 24 hours after injection for ^{125}I-bleomycin complex and ^{125}I-bleomycin A_2. Mice were sacrificed at 1, 6, 12, 24, 48 and 72 hours after injection with ^{114m}In-bleomycin A_2. A total of 6 mice were used at each time interval. Radioactivity in organs and tissues were expressed as percent dose per gram (% dose gm^{-1}). Radioactivity in the blood was expressed as % dose per 0.1 ml of whole blood (% dose 0.1 ml^{-1}). Tumor uptake was compared to uptake in muscle, blood and one other tissue which appeared to have significant uptake and which might be of clinical interest.

G. Whole Body Excretion Analysis of ^{125}I-Bleomycin A_2

Three mice were each administered 11 uCi of ^{125}I-bleomycin A_2 by tail vein injection. At various time intervals after injection the mice were placed in plastic bottles which were then placed into a small animal whole body counter. The radioactivity observed in the animals 5 minutes after injection was considered to be due to the entire dose injected. The amount of radioactivity remaining in the animals was determined by whole body counting at 1, 6, and 12 hours after injection then daily for 10 days. The counts were corrected for physical decay and expressed as a percentage of the activity observed in the animal at 5 minutes. An excretion curve was then plotted and analyzed.

RESULTS AND DISCUSSION

I. Chromatographic Properties of the Bleomycins, Iodinated Bleomycins, ^{114m}In-Bleomycin A_2, Na^{125}I and ^{114m}InCl$_3$

The relative migration rates of a group of structurally related compounds depends on their relative polarities in a chromatographic system. Polar substances migrate at slow rates while nonpolar substances migrate at faster rates. The degree of resolution attained will therefore depend on the relative differences in the polarity of the substances. Substances which are not structurally related can often be resolved on the basis of their solubility differences (93). For mixtures of structurally unrelated substances it is often possible to choose a chromatographic system in such a way that the principal components will be completely resolved, that is one substance will migrate with the solvent front while the other will remain at the origin (93).

The characteristic mobility of any particular substance in a given chromatographic system is defined by its reference or Rf value[a] The Rf value can not only be used to detect and help identify a substance, it can also be used to determine if the molecular structure of the substance has been altered in a significant way. Structural alteration of a molecule can significantly change its polarity and/or solubility characteristics thus causing the Rf of the substance to be displaced from its normal value.

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[a] $$\text{Rf} = \frac{\text{distance moved by the substance (cm)}}{\text{distance moved by the solvent front (cm)}}$$

The Rf value is characteristic for a given substance in a given chromatographic system.

Radiochromatography is an adaptation of traditional chromatography in that labeled compounds are developed on the chromatogram and then their radioactivity is used as a sensitive means of detecting the presence of the substance, localizing its position and giving a quantitative assessment of its concentration.

The technique of thin layer radiochromatography can be used in nuclear pharmacy to analyze the yields from radiolabeling procedures, for assessing the radiochemical purity of the product produced and often it can be used to determine if the reaction conditions have significantly modified the structure of the substance. Therefore, the behavior of the bleomycins, radiolabeled bleomycins and the radionuclides, $^{114m}InCl_3$ and $Na^{125}I$, was studied using a variety of chromatographic systems. The objectives were first, to develop an accurate and rapid method of assessing the presence, reaction yield and radiochemical purity of radiolabeled bleomycins and second, to determine if these labeled molecules were being grossly degraded by the reaction conditions.

A. Chromatographic Properties of the Bleomycins, Iodinated Bleomycins and $Na^{125}I$ on Silica Gel TLC Plates Using 10% Ammonium Acetate: Methanol (1:1)[a] as Solvent System

The Rf values obtained for each of the solutions is shown in Table 1. The unlabeled bleomycin complex was found to be resolved

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[a] 10% $NH_4OOCCH_3:CH_3OH$ (1:1) or 10% $NH_4Ac:MeOH$ (1:1).

Table 1

Chromatographic Properties of the Bleomycins, ^{125}I-Bleomycins and $Na^{125}I$ on Silica Gel TLC Plates Using 10% NH_4OOCCH_3:CH_3OH(1:1) As Solvent System

	Bleomycins							
	$A_2^{(b)}$	$B_2^{(b)}$	$A_2^{(c)}$	^{125}I-$A_2^{(d)}$	^{125}I-$B_2^{(d)}$	^{125}I-$A_2^{(e)}$	^{125}I-$A_2^{(f)}$	$Na^{125}I$
$Rf^{(a)}$	0.35 $\pm$	0.68 $\pm$	0.35 $\pm$	0.35 $\pm$	0.68 $\pm$	0.35 $\pm$	0.35 $\pm$	0.80-0.90
	0.01	0.00	0.01	0.01	0.01	0.01	0.01	

Development temperature - 20°C.
Development distance - 10 cm.

[a] Rf expressed as a mean of 3 values ± standard deviation

[b] Resolved from bleomycin complex (Blenoxane, Bristol Laboratories) and detected using short wave UV light.

[c] Bleomycin A_2 working standard

[d] ^{125}I-bleomycins were resolved from the iodinated complex.

[e] ^{125}I-bleomycin A_2 produced from iodination of A_2 working standard.

[f] ^{125}I-bleomycin A_2 produced from iodination of the A_2 fraction isolated from bleomycin complex.

into 2 fractions having Rf values of 0.35±0.01 and 0.68±0.00. These values are comparable to those reported by Umezawa (1), of Rf 0.40 for A_2 and Rf 0.68 for B_2. Umezawa obtained his Rf values using the copper containing compounds whereas this study used the copper-free bleomycin complex (Blenoxane, Bristol Laboratories). In addition, the exact composition of the stationary phase used in the study by Umezawa was not published.

The iodinated bleomycins were found to have the same Rf values as the unlabeled bleomycins. This is in agreement with the published values for other radiolabeled bleomycins (9)(11)(20). Mori (20), reported Rf values for ^{99m}Tc-bleomycin fractions which correspond to the values reported in this study. This indicates that the bleomycin molecule was not being grossly degraded by the reaction conditions in those experiments. In this study however, it was observed that Rf values obtained using ^{125}I-bleomycin produced by the chloramine-T method tended to be highly variable, relative to Rf values obtained from ^{125}I-bleomycin produced by the ICl method.

The iodinated fractions at Rf 0.35 and 0.68 were scrapped from the tlc plate and the radioactivity of each fraction was assayed in an appropriately calibrated gamma counter. The ratio of radioactivity in fractions A_2 and B_2 was found to be about 2:1. This is in agreement with the accepted composition of bleomycin complex (5), and also with results reported by Mori (20). It tends to confirm the belief that iodine does not have any preference for the A_2 fraction. Bleomycin A_2'-C, however has a histidine residue in its terminal amine position, giving it 2 possible iodination sites (24). Hence any appreciable quantity of this component in the bleomycin complex would tend to increase the radioactivity in the vicinity of the B_2 peak since A_2'-C has an Rf = 0.71 under the chromatographic conditions used in this study.

On silica gel tlc plates, and the solvent system previously described, $Na^{125}I$ was found to be a fast moving component migrating near the solvent front with an Rf of 0.85 The $Na^{125}I$ chromatogram was marked off in 1 cm segments, as previously described, the silica gel segments were individually scrapped from the plate into polyethylene counting vials and then assayed on an appropriately calibrated gamma counting system. Only 0.8±0.1% of the radioactivity was associated with the origin while 90% of the activity was found above Rf 0.75.

The silica gel tlc plates were satisfactory for determining Rf values, however they could not be used to assay the reaction yield as there was always found to be considerable tailing from the leading peaks making it impossible to decide where the division point was between the peaks. In addition, chromatograms required 2-2.5 hours to develop. The procedure of plate scrapping was unwieldy and time consuming and the friable layer tended to be a contamination hazard.

B. Chromatographic Properties of the Bleomycins, Iodinated Bleomycins and Na^{125}I on Eastman Silica Gel mylar backed 1x20 cm strips, #6061, Using 95% Ethanol as Solvent System

The Eastman #6061 sheets, as described previously, were cut into 1x20 cm strips. This medium was a convenient alternative to the more cumbersome tlc plates. However the bleomycin complex could not be satisfactorily resolved into separate fractions on Eastman medium in spite of many trials with different solvent systems. It was found, as shown in Table 5 that unlabeled bleomycins, with 10% NH_4OOCCH_3:CH_3OH(1:1) as solvent system, would migrate to a broad band between Rf 0.50-0.60. When 95% ethanol was used as solvent all bleomycins remained at the origin. The Na^{125}I, however, in 95% ethanol was a fast moving component with an Rf = 0.88 as shown in Table 2. As would be expected the ^{125}I-bleomycins also stayed at the origin under these conditions. Therefore, Eastman #6061 strips, using 95% ethanol as solvent was found to be an accurate method for determining the yield of the radioiodination reactions and for assessing the radiochemical purity of the products.

The main disadvantage with the Eastman system was slow development time. Like silica gel tlc plates, the Eastman strips required 2-2.5 hours to develop 13-15 cm. This is the distance that was found necessary to give adequate resolution between the labeled molecule at the origin and the highly mobile iodide peak at Rf = 0.88. This was especially true when the concentration of free Na^{125}I was relatively high because the peak tailed heavily into the regions between Rf = 0.50 and 0.80 unless the strip was developed 13-15 cm.

Table 2

Chromatographic Properties of the Bleomycins, ^{125}I-Bleomycins and Na^{125}I on Eastman #6061 Mylar Backed Silica Gel Strips

Using 95% Ethanol as Solvent System

	Bleomycin						
	A_2 (a)	B_2 (a)	^{125}I-A_2 (b)	^{125}I-B_2 (b)	^{125}I-A_2 (c)	^{125}I-A_2 (d)	Na^{125}I
Rf	0.00	0.00	0.00	0.00	0.00	0.00	0.80-0.90

Development temperature - 20°C
Development distance 13-15 cm.

(a) Resolved from bleomycin comples (Blenoxane, Bristol Labs) and detected with short wave UV light.

(b) Resolved from ^{125}I-bleomycin complex.

(c) ^{125}I-bleomycin A_2 working standard.

(d) ^{125}I-bleomycin A_2 isolated from bleomycin complex.

When $Na^{125}I$ alone was chromatographed on Eastman #6061 strips, less than 1% residual iodine was found to be retained at the origin.

Another minor disadvantage encountered with the Eastman system was the necessity of having to cut the chromatogram into 13 equal segments 1 cm long and assaying each one individually for radioactivity.

C. Chromatographic Properties of the Bleomycins, ^{125}I-Bleomycins and $Na^{125}I$ on Gelman ITLC(a) Silica Gel sheets Using 95% Ethanol as Solvent System

The chromatographic behavior of the bleomycin compounds and $Na^{125}I$ on Gelman tlc media is shown in Table 3. Their chromatographic behavior parallels, to a large degree, results obtained using Eastman chromatogram sheets. The labeled and unlabeled bleomycins failed to move from the origin while $Na^{125}I$ ran to the solvent front with an Rf centered at 0.90. The chromatograms on which $Na^{125}I$ alone was developed were cut into 1 cm segments and assayed for radioactivity in a suitably calibrated gamma counter. About 1% residual ^{125}I was found at the origin of the chromatograms.

The chromatograms developed on Gelman ITLC media, using a seprachrom(a) minitank and 95% ethanol as solvent were completely developed in 7 cm. This required about 7 minutes. The distribution of $Na^{125}I$ on Eastman and Gelman chromatograms is shown in Figure 5 and 6 and Appendix 2.

For Gelman ITLC, less than 3% residual ^{125}I was found in

(a) Trademark of Gelman Instrument Company, Ann Arbor, Mich.

Table 3

Chromatographic Properties of Bleomycins, ^{125}I-Bleomycins and $Na^{125}I$ on Gelman ITLC Sheets Using 95% Ethanol as the Solvent System

	Bleomycins						
	A_2[(a)]	B_2[(a)]	A_2[(b)]	^{125}I-A_2[(c)]	^{125}I-A_2[(d)]	^{125}I-A_2[(e)]	$Na^{125}I$
Rf	0.00	0.00	0.00	0.00	0.00	0.00	0.88-1.0

Development temperature - 20°C.
Development distance - 7 cm.

(a) Resolved from bleomycin complex (Blenoxane-Bristol labs) and detected with short wave UV light.

(b) Bleomycin A_2 working standard obtained from National Institute of Health of Japan, Tokyo, Japan.

(c) Resolved from ^{125}I bleomycin complex.

(d) ^{125}I-bleomycin A_2 working standard.

(e) ^{125}I-bleomycin A_2 isolated from bleomycin complex.

Distribution of Na^{125}I on Gelman ITLC

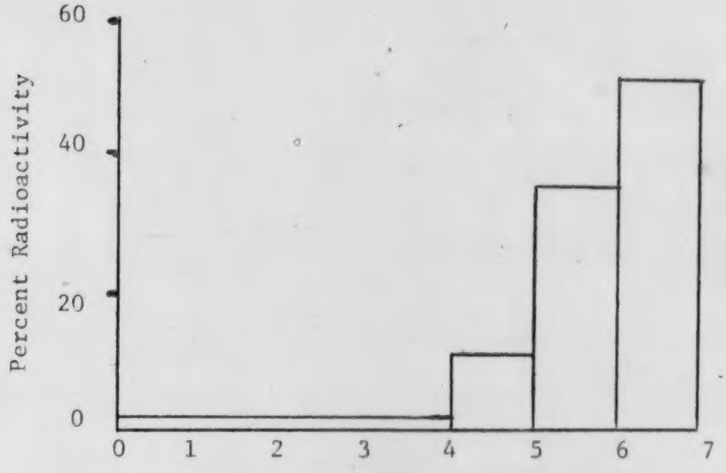

Figure 5

Distribution of Na^{125}I on Eastman Chromatograms

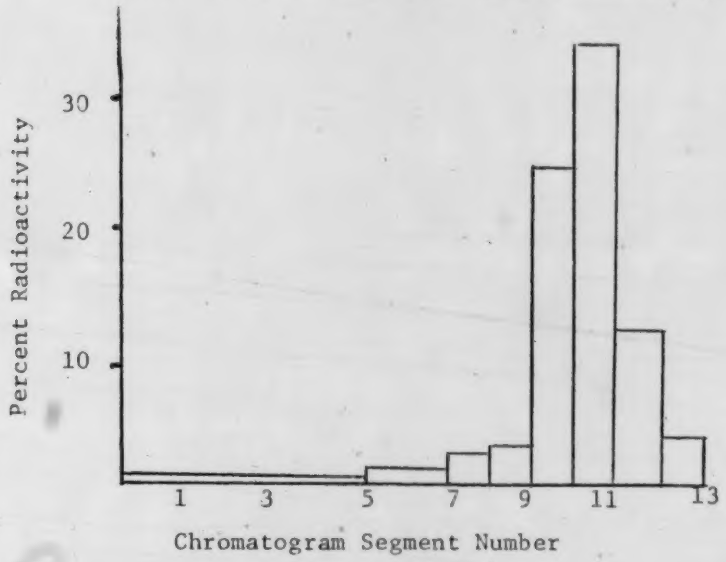

Figure 6

the chromatogram region Rf = 0 to Rf = 0.50. In contrast, Eastman chromatograms had about 10% ^{125}I distributed in this region. Therefore, quantitative estimates of reaction yields and radiochemical purity could be obtained by simply cutting the Gelman chromatograms into only 2 equal portions then assaying each half, that is one half containing the origin and the other half containing the solvent fronts in a suitably calibrated gamma counter.

The results of assays obtained on the Gelman system were compared to results obtained on Eastman chromatograms. Aliquots were removed, at various time intervals, from a series of iodination reactions and chromatographed using the 2 systems. The paired reaction yields thus obtained were subjected to a paired t-test and tested at the 5% level of significance (92). The results of the t-test, shown in Table 4, indicates that the paired yields were not significantly different and therefore Gelman and Eastman systems could be used interchangeably to quantitatively determine the yields of iodination reactions and to assess the radiochemical purity of the iodinated products.

D. Chromatographic Behavior of the Bleomycins, ^{114m}In-Bleomycin A_2 and $^{114m}InCl_3$ on Silica Gel TLC and Eastman Silica Gel Strips Using 10% $NH_4OOCCH_3:CH_3OH$(1:1) as Solvent System

The chromatographic properties of ^{111}In-bleomycin and $^{111}InCl_3$ have been well documented (9)(10)(11). The behavior of these substances on the chromatographic systems used in this study is shown in Table 5.

Table 4

Comparison of Reaction Yields(a) calculated from Assays Performed on Eastman Silica Gel Strips and Gelman Sheets Using 95% Ethanol as Solvent

	Yields(a)					
Experiment No.	1	2	3	4	5	6
Assay System:						
Gelman	73.7	80.4	82.9	74.8	72.9	76.9
Eastman	73.2	76.1	76.9	79.9	81.1	77.4
Difference	0.5	4.3	6.0	5.1	8.2	9.5

A paired t-test to determine if the observed differences in the assay results were significant was carried out after the method of Zalik (92). The calculated **t value** for the observed data was $t = 2.0$ while theoretical $t0.05$ value was $t0.05 = 2.8$ for 4 degrees of freedom.

Therefore, no significant difference was found between the two chromatographic systems for the reactions tested.

(a) Yields were calculated by expressing the radioactivity in counts per minute (cpm) as a percentage of the total radioactivity in cpm detected on the chromatogram.

Table 5

The Chromatographic Properties of Bleomycin A_2 ^{114}In-Bleomycin A_2 and $^{114m}InCl_3$ (at pH7)

Chromatographic System	RR Values $^{114m}InCl_3$ (pH 7.0)	In-Bleomycin A_2	Bleomycin$^{(b)}$ A_2	Solvent System
Silica Gel TLC plates	0.80-0.90	0.35±0.02	0.35±0.01	10% NH_4Ac:MeOH(1:1)$^{(a)}$
Eastman Silica Gel #6061	0.00-0.08	0.50-0.60	0.50-0.60	10% NH_4Ac:MeOH(1:1)$^{(a)}$
Eastman Silica Gel #6061	0.00-0.08	0.00	0.00	95% ethanol

Development temperature - 20°C.
Development distance 10 cm TLC 13-15 cm Eastman #6061

(a) 10% $NH_4OOCCH_3:CH_3OH(1:1)$

(b) Detected with short wave UV light.

The ^{114m}In-bleomycin A_2, on silica gel tlc, had an Rf of 0.35 which was identical to the value found for unlabeled bleomycin A_2. On Eastman silica gel strips #6061, bleomycin A_2 was detected as a broad band between Rf 0.50 and 0.60 when visualized under uv light. Identical chromatographic behavior was observed for the ^{114m}In-bleomycin A_2. The $^{114m}InCl_3$ under neutral conditions was hydrolyzed to $^{114m}In(OH)_3$ which was found to be insoluble and which stayed at the origin of the chromatogram.

Therefore, silica gel tlc provided a means of identifying the radiolabeled bleomycin and for determining if any gross changes had occurred in its molecular structure during the reaction. The Eastman silica gel system, using the same solvent system as silica Gel tlc, provided a means of assessing the reaction yield and the radiochemical purity of the labeled product.

E. Chromatographic Behavior of the Bleomycins, ^{125}I-Bleomycins and Na^{125}I on Sephadex G-10 Columns

Gel filtration chromatography is a preparative and analytical technique that is used to separate molecules according to size (94). Molecules larger than the exclusion limit (the largest pores of the swollen sephadex) cannot penetrate the gel particles and therefore these molecules will pass through the gel bed, in the liquid phase outside of the sephadex particles and are eluted from the column first. Smaller molecules penetrate the gel particles, to varying degrees, depending on their size and shape (94). Molecules are therefore eluted from a sephadex bed in order of decreasing molecular size. Thus sephadex G-10 columns can be used to purify reaction mixtures by excluding a large molecule, such as ^{125}I-bleomycin, so that it is eluted first, while retarding small inorganic impurities such as ^{125}I ions. By proper control of column length and the flow rate of the eluate a sample can be eluted in a volume only slightly larger than its volume before it was applied to the top of the sephadex column (94).

On small sephadex G-10 minicolumns, prepared as previously described, the labeled and unlabeled bleomycins were found to be eluted at the column void volume while free $^{125}I^-$ was eluted in a band at twice the void volume.

The minicolumns were used primarily to assess the degree of progress of radioiodination during labeling experiments. A 10-20 μl aliquot of the reaction mixture was eluted through the column, using water or 0.01 M buffer as eluant, and the radioactivity was detected by a 3"x3" shielded NaI(Tl) crystal. A radioactive peak at the column void volume was indicative of the presence of ^{125}I-bleomycin in the reaction mixture. Blank reactions (no bleomycin in the reaction mixture) were treated in parallel with all reaction runs and aliquots of the blanks were assessed on G-10 minicolumns in parallel to ensure that the peak at the void volume was not due to polymer formation or to contamination in the buffer system.

A reasonably rapid method of quantitating the reaction yield on sephadex G-10 minicolumns, consisted of collecting the ^{125}I-bleomycin and free ^{125}I peaks separately, measuring their volumes then assaying the radioactivity in an aliquot from each peak and correcting for dilution. This would give a measure of the relative counts associated with each peak thus providing a rough measure of reaction yield. A 100% elution from the column was assumed. The results obtained were only approximate because the ^{125}I-bleomycin peak always contained at least 10-15% of its radioactivity as free ^{125}I. This was confirmed on numerous occasions by radiochemical assays performed on Eastman #6061 silica gel strips. This free ^{125}I in the ^{125}I-bleomycin fraction could have been due to the short column length (4-6 cm) and the high flow rate of the mobile phase. However, good resolution of the ^{125}I-bleomycin and $^{125}I^-$ peaks was always obtained and it was felt that the free $^{125}I^-$ at the void volume could also be due to dissociation of weak nitrogen-iodine bonds as the reduced reaction mixture was eluted through the column. The sephadex matrix also contains a small number of carboxylic groups, which in a low ionic strength solvent system, can interact with negatively charged substances excluding them from the gel phase. The negative substance may then be eluted at or near the column void volume (94). This effect can be eliminated by the use of solvent systems with ionic strengths of at least 0.02M. The radiochemical purity of the products eluted from the G-10 minicolumns therefore, was not found to be satisfactory for in vivo studies.

Further experimentation indicated that it was possible to pass the sephadex G-10 eluate through a small Dowex 1x4 layer to reduce the residual ^{125}I to a satisfactory level. Radiochemical purity of the Dowex 1x4 eluate was found to be greater than 93%.

However, the G-10 minicolumns diluted the volume placed on top of the sephadex column by a factor of 20 and this dilution factor was found to be unacceptable since once ^{125}I-bleomycin was placed in an aqueous solvent it became difficult to extract and the diluted radioactive solution could not be used satisfactorily in vivo because of its low radio concentration. Therefore it seemed feasible to use a mixed column containing a relatively large volume of G-10 on top with a small 1-2mm layer of Dowex 1x4 on the bottom of the column to give a one step purification technique. To decrease the band broadening effect that occurred on G-10 minicolumns longer (15 cm) sephadex columns and slower elution rates were tried. Longer columns and slower elution rates however tended to defeat the objectives of the study which were to develop rapid production, purification and assay procedures so that short-lived ^{123}Iodine could be used to label bleomycin. In addition to increased elution times, long columns tended to retain variable, but sometimes significant amounts of radioactivity. This is probably related to the carboxylic groups in the gel matrix which under conditions of low ionic strength, tend to adsorb cations. A significant proportion of the bleomycin, and presumably ^{125}I-bleomycin, would be cationic at pH7 and they could be adsorbed in increasing quantities as the length of the sephadex column increased. No attempt was made to determine if the retained radioactivity was due to free $^{125}I^-$ or ^{125}I-bleomycin.

Therefore, Sephadex G-10, when used in the manner described in the experiments, was found to be satisfactory only as a means of determining if ^{125}I-bleomycin was present in a reaction mixture. The Sephadex G-10 procedure, as used, was unsatisfactory because it unduly diluted the fraction being eluted, it did not provide an accurate method for assaying the yield of a reaction and it could not be used alone to purify the ^{125}I-bleomycin reaction mixture for in vivo studies.

II. Isolation and Purification of Bleomycin A_2 From the Commercially Available Bleomycin Complex[a]

In order to be reproducible, biological and chemical studies must be done with compounds of known composition. In this way the unique properties of each compound can be defined and assessed. Bleomycin A_2 is the most abundant bleomycin fraction in the commercially available complex (Blenoxane, Bristol Laboratories, Candiac, P.Q.). Preliminary animal studies (4)(5), have shown that bleomycin A_2 may have some potentially useful biological properties. For example, bleomycin A_2 was found to be rapidly excreted from most non-tumor tissues but at the same time it was found to have a rapid and selective uptake into some animal tumors with a subsequent slow rate of degradation and excretion. Therefore, in an effort to achieve increased tumor specificity, a number of experiments were undertaken to isolate the A_2 fraction from the bleomycin complex and to study the tumor affinity of the bleomycin A_2 fraction.

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[a] Blenoxane, Bristol Laboratories, Candiac, Que. (Canada).

In a preliminary experiment 0.80 mg of bleomycin A_2 working standard (National Institutes of Health, Tokyo, Japan) was applied, in 0.1 ml of water, in a band along the origin of a 5x20 cm silica gel tlc plate and developed in 10% $NH_4OOCCH_3:CH_3OH(1:1)$. The bleomycin A_2 appeared as a wide band centered at Rf 0.35 when viewed under short wave uv light. This band was scraped from the plate and the bleomycin A_2 was extracted from the silica gel scrapings with methanol as previously described. The quantity of bleomycin A_2 in the eluate was determined from a standard curve, as shown in Appendix I. It was found that 0.55 mg, or about 67%, of the initially applied amount of bleomycin A_2 could be recovered by this technique.

After the trial isolations of bleomycin A_2 were found to be feasible, entire vials of bleomycin complex (Blenoxane, Bristol Laboratories, Candiac, P.Q.) were similarly processed. The contents of 1 vial of bleomycin complex was dissolved in 0.5 ml of water and then applied as a band along the origin of a 20x20 cm silica gel tlc plate and developed using 10% $NH_4OOCCH_3:CH_3OH(1:1)$ as solvent system. The silica gel containing the bleomycin A_2 was scraped from the plate and extracted with methanol. The methanol containing the bleomycin A_2 was evaporated to dryness and the residue taken up with appropriate buffer. The bleomycin A_2 was then used for radioiodination experiments.

Three batches of bleomycin A_2 were isolated from vials of the commercially available bleomycin complex. The results are shown in Table 6.

Table 6

Quantity of Bleomycin A_2 Recovered from the Commercially Available Bleomycin Complex (Blenoxane, Bristol Labs.)

Vial	Net Weight of Contents	Recovery of A_2 Fraction
1	8.2 mg	2.6 mg
2	8.2 mg	2.1 mg
3	8.3 mg	2.4 mg

Average recovery of bleomycin A_2 = 2.4 ± 0.3 mg.

The bleomycin A_2 fraction, as recovered from the bleomycin complex, could be used for iodination reactions without further purification. However, the bleomycin A_2 still contained residual ammonium acetate from the buffer system and unknown impurities from the silica gel. Bleomycin A_2 was further purified when it was used for biological studies. Purification was accomplished by extracting the bleomycin A_2 from the aqueous alcoholic solvent system with Amberlite IRC-50 cation exchange resin (in the H^+ form) for 2 hours.

The cation resin containing the adsorbed bleomycin A_2 was separated from the supernatant which would contain most of the impurities. The bleomycin A_2 was eluted from the Amberlite IRC-50 using methanol acidified to pH 4.6 with 0.1N HCl. The extraction of bleomycin A_2 from the Amberlite IRC-50 resin was monitored by a flow cell in a uv spectrophotometer (Beckman Model DB) at 254 nm.

When all the bleomycin A_2 was extracted from the resin the methanol was neutralized and the solution evaporated to dryness over low heat. The bleomycin A_2 was taken up into a minimum quantity of warm ethanol. The solution of bleomycin A_2 in ethanol was placed into a larger vial which contained a small volume of diethyl ether. The larger vial was tightly sealed and allowed to stand at room temperature. The ether vaporized and mixed with the alcohol thus changing the polarity of the solvent system containing the bleomycin A_2. The bleomycin A_2 would crystallize out, usually within 24 hours. Appreciable amounts of water in the solvent system made crystallization difficult and recovery highly variable.

In order to confirm the recovery of bleomycin A_2 from the IRC-50 resin, 0.80 mg of bleomycin A_2 working standard was dissolved in 15 ml of methanol and extracted with Amberlite IRC-50 as previously described. The Amberlite IRC-50 resin containing the adsorbed bleomycin A_2 was eluted with acidified methanol. The recovery of bleomycin A_2 was found to be 0.70 mg or about 87%. When the bleomycin was recrystallized using the ethanol-ether solvent system 0.52 mg was recovered although these recoveries varied widely, possibly because of excess water in the solvent system.

Therefore, the net recovery of bleomycin A_2 from the bleomycin complex, if all purification steps were done, was estimated to be: 67% recovery from the silica gel scraping, 87% from the IRC-50 resin and about 60% from recrystallization or a net recovery of 35%. However, an extremely high quality of bleomycin A_2 fraction could be produced by this method.

The procedure outlined was tedious, time consuming and not very efficient. Recently, a new technique has appeared in the literature for separation of bleomycin fractions (47). This method uses high performance liquid chromatography (hplc) with Porasil A as stationary phase and 0.3% $NH_4OOCCH_3:CH_3OH$(1:1) as mobile phase. The use of this technique as a preparative method for isolating bleomycin A_2 warrants further investigation. Alternately, it could possibly be used for the preparative separation of ^{125}I-bleomycin A_2.

III. Radioiodination of Bleomycin A_2: A Comparison of the Chloramine-T and the Iodine Monochloride Iodination Methods

A. Radioiodination of Bleomycin A_2 Using the Chloramine-T Method

There are two general approaches to labeling proteins and polypeptides using chloramine-T. The original approach of Hunter and Greenwood (66), used high chloramine-T concentrations and very short reaction times. A modification of this approach developed by McConahey *et al*. (89), used much lower chloramine-T concentrations and reaction times up to 5 minutes. Both of these methods were capable of producing high specific activity radioiodinated proteins.

1. The Effect of Chloramine-T Concentration on the Reaction Yield of ^{125}I-Bleomycin A_2

Proteins and high molecular weight polypeptides usually contain a number of readily available reducing groups, such as cystine, cysteine and tryptophane residues. These peptide residues are easily oxidized by the chloramine-T thereby reducing the quantity of oxidizing agent that is available for producing cationic iodine. Since the number of these reducing groups varies with the specific protein or polypeptide, the amount of oxidizing agent that will be required to achieve a satisfactory level of iodination will also vary accordingly. In addition, the empirical evidence of Hunter (86), suggests that the amount of chloramine-T might also depend on the concentration of radioactive iodine in the reaction mixture since trace quantities of ^{125}I invariably required increased concentrations of chloramine-T to maintain iodination efficiency.

In the present set of experiments a large range of chloramine-T concentrations were used initially so that the optimal value for radiolabeling would, at least, be indicated. The concentrations used were 40, 80, 300 and 800 ug of chloramine-T per reaction mixture. This would give a set of chloramine-T-to-iodide and chloramine-T-to-peptide ratios that would be comparable to the ratios used by McConahey at one extreme and Hunter at the other extreme. A reaction time of 120 seconds was initially chosen because it was also intermediate between the reaction times of these two extreme approaches. A pH of 7.8 was used because it was generally accepted as being at or near the optimal pH for the iodination of proteins (66)(86).

The yields of ^{125}I-bleomycin A_2 obtained with these initial reaction conditions are shown in table 7. The optimal concentration of chloramine-T in the reaction mixture was found to be 800 ug which gave an average yield of about 26%. The 40 and 80 ug quantities of chloramine-T gave only trace labeling. With 300 and 800 ug quantities of chloramine-T, significant yields of ^{125}I-bleomycin A_2 were obtained when compared to blank reactions which contained all the reaction ingredients except the bleomycin. The optimum concentration of chloramine-T required in these experiments was more than the 100-200 ug used by McConahey et al. (89) but less than the 1000 ug quantities originally used by Hunter (66).

Table 7

The Effect of Chloramine-T Concentration and Temperature on the Reaction Yields of ^{125}I-Bleomycin A_2 (a)

Chloramine-T Concentration in ug	Bleomycin Concentration in ug	Temperature °C	Reaction Yield (b) Experiment Number 1	2	3	4	Average
40	164	20	-	-	-	-	-
80	164	20	- - - trace - - -				
300	164	20	12.6	15.1	15.9	20.9	16.1±1.7
800	164	20	33.1	29.0	19.2	26.4	26.9±5.9
800	-	20	1.6	1.2	2.8	0.8	1.5±1.0
-	164	20	3.2	4.8	5.6	3.1	4.2±1.2
800	164	0	16.6	24.3	21.6	26.4	22.2±4.2

(a) Bleomycin A_2 was isolated from bleomycin complex (Blenoxane, Bristol Labs., Candiac, P.Q.).

(b) All reactions contained 20 uCi of ^{125}I. Yields were obtained from Eastman chromatograms developed using 95% ethanol. Yield was calculated by expressing the activity (cpm) at origin as a percent of total activity (cpm) on entire chromatogram.

It is well known that high concentrations of chloramine-T may induce subtle chemical changes into the structure of the molecule being iodinated and these structural changes could modify the biological behavior of the compound. The oxidizing action of chloramine-T then, could destroy or modify the tumor specificity of bleomycin. Therefore it was felt that any attempts to increase the reaction yield of ^{125}I-bleomycin A_2 should not be done by merely increasing the chloramine-T concentration but rather by attempting to identify and to optimize the other parameters that could influence radioiodination yield.

2. The Effect of pH on the Reaction Yield of ^{125}I-Bleomycin A_2

The effect of pH on reaction yield in this set of experiments is shown in Table 8. The maximum yield of 20.3% ^{125}I-bleomycin A_2 was found to occur at pH 7.5 with yields falling off sharply in the extreme alkaline ranges tested. The shape of the curve appears to be comparable to the one obtained by Hunter for the radioiodination of proteins (86).

A Duncan's multiple range test was carried out on the mean reaction yields at the 5% level of significance, after the method of Zalik (92). A Duncan's multiple range test is a method of comparing a set of treatment means. The hypothesis being tested is that the set of all treatment means are equal. It is used in place of an F-test when there are more than 2 treatments. In the test, the difference between the ranked means is compared with a set of standard significant differences, derived from appropriate tables (92). The size of the standard significant differences depend, in part on the closeness of the means after ranking (being smallest for adjacent means and largest for the extremes) and in part on the standard deviation of the means (92). The difference between means is significant if the observed difference between the means is greater than the theoretical standard significant difference for the ranks being compared (92).

Table 8

The Effect of pH on Reaction Yields of ^{125}I-Bleomycin

	Experiment Number[a]			
pH	1	2	3	Average
6.0	13.2	12.6	16.1	14.0±1.9
7.0	16.4	17.1	18.2	17.2±0.3
7.5	17.0	21.7	22.3	20.3±2.9
8.5	11.8	10.9	9.9	10.9±0.3
9.0	10.1	9.9	9.7	9.9±0.0

[a] All radioiodinations were carried out at 20°C with a reaction time of 60 seconds.

The results of the Duncan's test is that the average reaction yields of ^{125}I-bleomycin A_2 obtained at pH 6.0, 7.0 and 7.5 were all significantly different from one another and from the reaction yields obtained at pH 8.5 and 9.0. The reaction yields obtained at pH 8.5 and 9.0 were not significantly different from each other. Alkaline borate buffer was used to supply reaction mixtures with pH 8.5 and 9.0 while more acidic pH values were supplied by 0.5 M phosphate buffers (90). Since the alkaline borate buffer was used extensively in the ICl radioiodination procedure, to be described, the depressed yields of ^{125}I-bleomycin observed at the more alkaline pH values was not attributed to the buffer. Instead the depressed yields were assigned to the conversion of iodide to iodate which is known to occur rapidly at alkaline pH values in the presence of an oxidizing agent (66)(70)(71)(86).

3. The Effect of Temperature on Reaction Yields of ^{125}I-Bleomycin A_2

The rationale for carrying out iodination reactions at low temperatures is based largely on theoretical considerations. Low temperatures may slow the rate of iodate formation (71). Low temperatures may also protect the integrity of the compound by slowing the rate of oxidation of any labile groups which might be present in the molecule.

The effect of temperature on reaction yield is shown in Table 7, Page 99 . There was no significant difference between yields of ^{125}I-bleomycin A_2 obtained at 20°C and at 4°C. When the reaction mixtures were chromatographed on silica gel tlc plates, using 10% $NH_4OOCCH_3:CH_3OH(1:1)$ as solvent, and viewed under uv light, Rf values of 0.35 were obtained. The plates were marked off into 1 cm segments and then each segment was assayed for radioactivity. Significant radioactivity was detected at Rf 0.35 and 0.75-0.85 corresponding to the peaks for ^{125}I-bleomycin A_2 and $Na^{125}I$ respectively. There was no difference in the chromatographic behaviors of the 2 reaction mixtures and therefore it was decided, for convenience, to carry out all reactions at 20°C.

4. The Effect of Reaction Times on the Yields of ^{125}I-Bleomycin A_2

Hunter and Greenwood (66) in their original chloramine-T radioiodination procedures, used very short reaction times to minimize the exposure time of the protein to the damaging effects of the oxidizing agent. McConahey et al. (86), later showed that reduced concentrations of chloramine-T and prolonged reaction times could be used to iodinate a variety of proteins without significant evidence of damage to the molecule. In this study iodination reactions were carried out for time intervals from 30-300 seconds as shown in Table 9. Only trace labeling was observed for the 30 second reaction time. Reaction yields improved significantly up to 29.6% at 180 seconds and then appeared to fall slightly at a reaction time of 300 seconds. The reaction yield of 28.6% at 120 seconds was not significantly different from that obtained at 180 seconds and subsequently all radioiodination reactions using chloramine-T were carried out at a reaction time of 120 seconds.

These series of experiments identified chloramine-T concentration, pH and reaction time as the parameters which could influence the degree of radioiodination of bleomycin. In order to secure the maximum yield of ^{125}I-bleomycin A_2, with minimal molecular degradation, these parameters should be optimized for the lowest concentration of chloramine-T that is consistent with satisfactory levels of radioiodination. Unsatisfactory yields of ^{125}I-bleomycin A_2 however, were obtained throughout these chloramine-T experiments.

Table 9

The Effect of Reaction Times on ^{125}I-Bleomycin A_2 Reaction Yields

	Reaction Yields (%)					
	Experiment Number					
(seconds)	1	2	3	4	Blank[a]	Average Yield[b]
30	5.0	3.1	8.2	6.1	1.6	5.6±2.1
60	16.6	12.8	19.2	17.1	3.4	16.4±2.7
120	27.8	31.6	29.4	25.6	2.9	28.6±2.5
180	33.8	27.6	28.5	28.3	4.7	29.6±2.9
300	29.8	19.2	27.6	23.3	5.9	25.0±4.7

[a] Blank reactions, contained all ingredients except bleomycin A_2.

[b] Mean of 4 reaction yields ± standard deviation.

The low yields were probably the result of a combination of adverse competing reactions which consumed chloramine-T. The reactions consuming chloramine-T and adversely effecting radioiodination of bleomycin A_2 would be formation of unstable nitrogen-iodide bonds which would dissociate in the presence of mild reducing agents such as sodium metabisulfite or excessive oxidation of labile groups in the bleomycin molecule. In the latter case, the biological behavior of ^{125}I-bleomycin A_2 may be different from the bleomycin A_2.

Radioiodination of bleomycin complex produced yields which were not significantly different from the yields obtained from the radioiodination of bleomycin A_2 and therefore to report these results would be redundant.

B. Radioiodination of Bleomycin A_2 Using the Iodine Monochloride Method

Since the chloramine-T method of radioiodination of bleomycin A_2 did not produce satisfactory yields, an alternative milder method of radioiodination was sought which would give adequate yields of ^{125}I-bleomycin A_2.

The iodine monochloride method of McFarlane (65) had proven to be both a mild and a consistent method of introducing radioactive iodine into polypeptides and proteins. Inactive carrier ICl interacts with carrier free ^{125}I to give a dipolar radioactive iodinating species in which the iodine is thought to carry a positive charge. When the concentration of iodine is small relative to the concentration of ICl, virtually all of the radioactive iodine is found incorporated into ICl which can then interact with the substrate, as previously described in the literature survey.

Molecular iodine, in aqueous acidic media is a mild oxidizing agent (71)(74)(76), whose oxidation potential decreases as the pH of the solution is raised to neutrality. Therefore, while the oxidizing power of molecular iodine is still present at neutral pH it is attenuated and could be reduced even further if the concentration of ICl required for efficient labeling could be kept low.

1. The Effect of ICl Concentration on the Yield of ^{125}I-Bleomycin A_2

Hung (76), has shown that the half-life of ICl in aqueous solutions at pH 7.4 was about 10 minutes. Therefore, it seemed logical to use low concentrations of ICl and to add small aliquots every 10 minutes to maintain the concentration of ICl at levels that would give satisfactory iodination yields without damage to the bleomycin molecule. Hung (76), had previously obtained virtually 100% incorporation of iodine into low molecular weight model peptides using ICl/peptide molar ratios of 0.25 with molar ratios of 0.5 and 1 being required if the peptide contained easily oxidizable groups or if the imidazole ring of the histidyl residue was partially blocked or hindered.

Reif (69), on the other hand, had found that the iodination of a high molecular weight protein containing many tyrosyl residues required the use of ICl/protein molar ratio of 16 to obtain satisfactory incoproration of iodine into protein. However in other low molecular weight proteins, containing a limited number of iodination sites, the yield obtained was essentially a linear function of the ICl/protein molar ratio up to 4 after which the efficiency of iodine incorporation into the protein declined significantly.

In a series of radioiodination experiments, a wide range of ICl concentrations were used to determine the ICl/bleomycin molar ratio that would give maximum yields of ^{125}I-bleomycin A_2. The results of these experiments is shown in Table 10. Maximum yields of ^{125}I-bleomycin A_2 were obtained at ICl/bleomycin molar ratios of 1.0 to 3.5. Reaction yields were significantly depressed when molar ratios were 0.5 or 10.0.

Table 10

Reaction Yields of ^{125}I-Bleomycin A_2 as a Function of ICl Concentration

ICl/Bleomycin A_2 Molar Ratio	Reaction Yield at t=20 minutes
0.5	56.5±2.5
1.0	78.4±1.6
1.5	81.2±2.4
3.5[a]	77.6±0.9
10[a]	41.5±2.6

[a] ICl/bleomycin A_2 ratios were adjusted by decreasing the quantity of bleomycin A_2 in the reaction mixture thereby keeping the volume of the reaction mixture constant.

The optimum ICl/bleomycin A_2 ratio was found to be at 1.5 but yields obtained at this ratio were not significantly different from yields obtained at ICl/bleomycin molar ratios of 1.0 and 3.5. This is generally in agreement with the results of Hung and Reif which indicate that the efficiency of radioiodination reactions are decreased at both high and low ICl/peptide molar ratios.

2. The Effect of pH on the Yield of ^{125}I-Bleomycin A_2

The effect of pH changes on the yield of ^{125}I-bleomycin A_2 reactions was not attempted experimentally since theoretical considerations and the experimental results obtained from other studies (58)(69)(70)(71)(76), indicate strongly that the optimal pH for the radioiodination reactions of peptides and proteins must lie in a narrow range between pH 6.5 and 7.5. It has been established by Hung (76), that histidine was only slowly iodinated at pH 4 while at pH 9-10 the half-life of the iodinating species was decreased with a concomitant increase in the rate of formation of stable iodate.

3. The Effect of Reaction Time and Temperature on Yields of ^{125}I-Bleomycin A_2

A series of radioiodination reactions were carried out at 20°C. At regular intervals aliquots of the reaction mixture were removed and assayed, as previously described, on Eastman and Gelman chromatographic systems. A series of identical reactions were carried out at 4°C and allowed to proceed for 20 minutes before the reaction was terminated with sodium thiosulfate. The result from this series of reactions is shown in Table 11
There was no significant difference found between the reactions carried out at 4°C and those at 20°C and therefore all subsequent reactions were carried out at 20°C for convenience. The maximum yield of 80.4% was obtained with a reaction time of 20 minutes in a reaction mixture containing a ICl/bleomycin ratio of 1. The reaction yields were not increased significantly even after reaction times of 120 minutes.

The reaction mixtures were chromatographed on silica gel tlc using 10% NH_4OOCCH_3:CH_3OH(1:1) as solvent system and viewed under uv light. Bleomycin A_2 was vizualized at Rf 0.35, and no other spots were detected. The silica gel plates were divided into 1 cm segments and each segment was assayed for radioactivity

Table 11

The Effect of Reaction Time and Temperature on the Reaction Yields of ^{125}I-Bleomycin A_2 Using ICl Method of Radioiodination

Reaction time (minutes)	Blank(b) Reaction	Reaction Yields of ^{125}I-Bleomycin A_2 (%)(a)			
			ICl/Bleomycin Molar Ratios		
		1.0 0°C	1.0 20°C	3.5 20°C	10.0 20°C
0	0.9±0.2		6.2±0.4	5.9±0.8	5.6±0.8
3	2.8±0.4		38.8±1.4	29.4±4.1	19.4±3.6
10	4.1±0.4		73.7±3.2	69.9±2.3	28.6±1.1
20	4.9±0.3	76.3±2.1	80.4±0.8	77.6±0.9	41.5±2.6
30	7.3±0.4		80.7±1.8	75.6±2.3	39.5±2.7
60	8.2±0.7		76.1±4.1	73.7±3.2	42.6±1.4
90	6.3±0.6		87.7±0.8	76.5±2.6	43.4±1.7
120	5.8±0.8		78.2±2.0	75.4±2.6	48.7±1.5

(a) Expressed as mean of 3 reactions ± standard deviation. Radioiodination yields were determined from Gelman chromatographic systems using 95% ethanol as solvent. Yields were calculated as the activity detected at origin expressed as a percent of total activity detected on entire chromatogram. The yields were confirmed using Eastman chromatographic system.

(b) Blank reaction contains all ingredients except bleomycin.

as previously described. Radioactivity was detected at Rf 0.35 and 0.75-0.85 corresponding to ^{125}I-bleomycin A_2 and free Na^{125}I respectively. Therefore, no changes in the chromatographic behavior of the compounds were observed and extensive molecular degradation probably did not occur under the reaction conditions employed in these studies. However, about 20% of the iodine initially present remained unreacted after 120 minutes which may indicate that the iodinating species was being consumed by nitrogen-iodide bond formation or by reducing groups present in the bleomycin molecule. If the latter was true then the bleomycin A_2 molecule was probably undergoing subtle chemical modification which would not be detected chromatographically.

IV. Quality Control of ^{125}I-Bleomycin A_2

The radiochemical purity of a radioactive material may be defined as the proportion of the total radioactivity that is in the stated chemical form (95).

Radiochemical impurities, in solutions of radioactively labeled compounds, can arise when the labeling radionuclide exists in a free or unattached state or when radioactive species arise from the oxidative or hydrolytic cleavage of the radioactive compound. Organic radioactively labeled chemicals can undergo decomposition by any of the mechanisms known to effect non-radioactive substances but in addition they can undergo significant decomposition under the influence of their own radiation.

Therefore, the radiochemical purity of a radioactive compound is a manifestation of its general chemical stability. This is important when biological studies are undertaken since the presence of radiochemical impurities can often hinder the observation of the in vivo effects of the radiolabeled compound under investigation.

A. Radiochemical Purity of ^{125}I-Bleomycin A_2

1. The Presence of Free Na^{125}I

Eastman chromatogram #6061 strips and Gelman ITLC sheets were found to give comparable, reproducible and quantitative resolution of the ^{125}I-bleomycin A_2 and the Na^{125}I components in a reaction mixture when 95% ethanol was used as solvent system. Therefore both chromatographic systems could be used to assess the radiochemical purity of ^{125}I-bleomycin A_2 solutions.

Several methods were used to reduce free Na^{125}I in ^{125}I-bleomycin A_2 solutions to an acceptable 5% level. Of these, only 1 method, employing Dowex 1x4 anion exchange resin, proved to be satisfactory. Two methods, a solvent extraction technique and sephadex G-10, gave unacceptable results however both warrant further investigation while a fourth approach, using a cation exchange resin, was totally unacceptable.

a. Purification of ^{125}I-Bleomycin A_2 Solutions Using Dowex 1x4 Anion Exchange Resin

Thin, 2 mm to 0.5 cm layers of hydrated, Dowex 1x4 anion exchange resin were placed between frittered glass discs fitted into 25 cm glass syringes. The excess water from the resin bed was forced out by depressing the syringe plunger then the ^{125}I-bleomycin A_2 reaction mixture was allowed to percolate into the gel bed. The flow was controlled by a clamp and a small section of polyethylene tubing attached to the orfice of the syringe barrel. Flow rate was kept as low as practicable, usually at 0.1 ml/minute. When iodination reactions containing trace (20-40 uCi) quantities of ^{125}I were purified, small layers of Dowex 1x4 resin, 2-4 mm thick, could be used to reduce free iodide levels in the eluate to 2-3%. The gel bed was washed with 0.1 ml of water after the reaction mixture had passed through, to remove any ^{125}I-bleomycin A_2 solution trapped in the gel matrix. A Dowex bed size of 0.5 cm was found to be needed when ^{125}I-bleomycin A_2 reaction mixtures containing 2-3 mCi of Na^{125}I were being purified. Generally, the results obtained depended on the flow rate of the reaction mixture through the gel bed. For the thick gel beds, the reaction mixture could be percolated into the matrix and allowed to equilibrate 10-15 minutes before being eluted. However, a 0.5 cm gel bed usually retained 10-15% of the volume of the reaction mixture and therefore it was more satisfactory to pass the reaction mixture 2 or 3 times through a thin gel bed. In all cases, the free Na^{125}I activity was reduced to less than 5% of the total radioactivity in the ^{125}I-bleomycin A_2 solution. This method of removing unreacted ^{125}I was used for subsequent preparation of ^{125}I-bleomycin A_2 and ^{125}I-bleomycin complex for animal distribution studies.

A series of reaction mixtures, produced by the iodine monochloride method, were purified on Dowex 1x4 layers, as previously described, and the results are shown in Figure 7 and 8 and Tables 12 and 13.

^{125}I-Bleomycin A_2 Before Dowex 1x4 Treatment

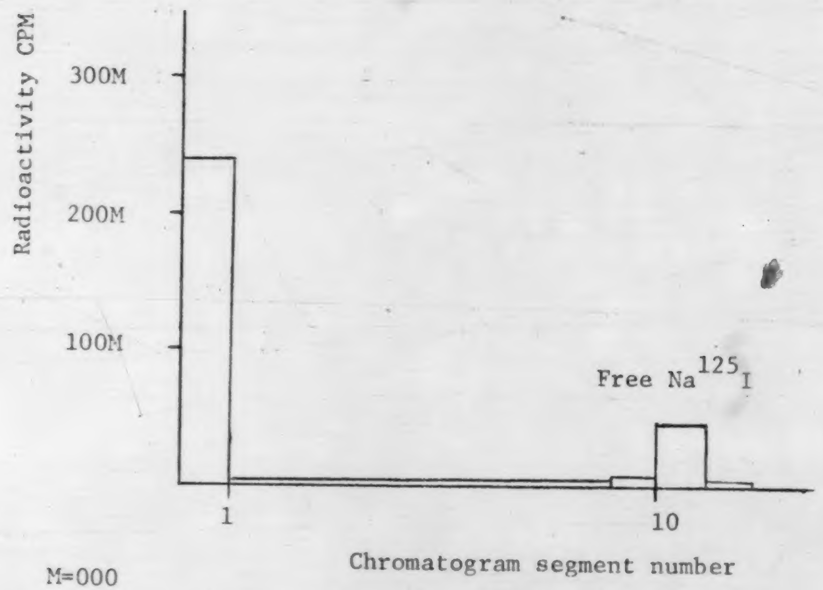

Figure 7

^{125}I-Bleomycin A_2 After Dowex 1 x 4 Treatment

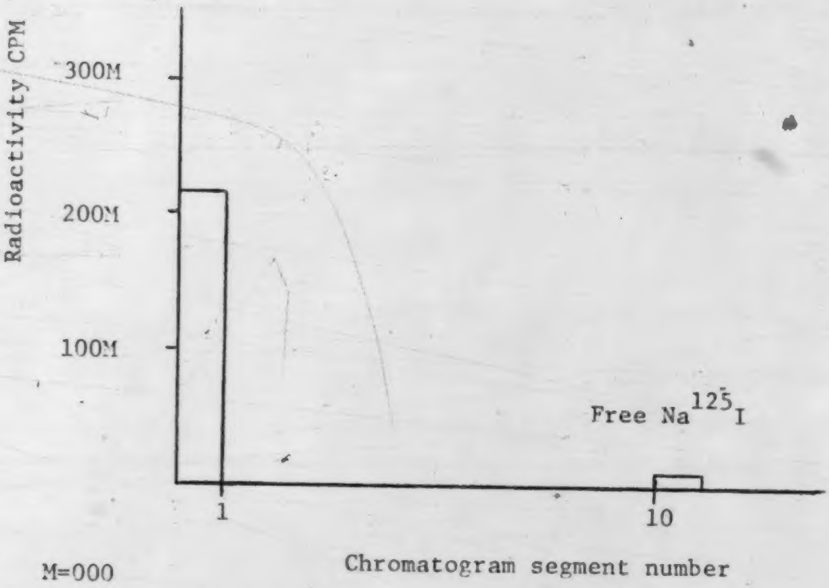

Figure 8

Table 12

^{125}I-Bleomycin A_2 Reaction Mixture(a) Before Removal of Free Na^{125}I

	Eastman #6061 System(b)			Gelman ITLC System(b)		
Reaction(c)	Total CPM on Chromatogram	CPM at Na^{125}I Peak	% Free Na^{125}I	Total CPM on Chromatogram	CPM at Na^{125}I Peak	% Free Na^{125}I
1	288817	47612	16.5	293409	45421	15.5
2	281419	39065	13.9	280642	38118	13.6
3	292719	43614	14.9	290905	44085	15.2
4	303412	59681(d)	19.7	298618	54218	18.2

(a) 10 ul of reaction mixture was diluted to 1 ml and 10 ul of this dilution was chromatographed on Eastman #6061 and in triplicate on Gelman ITLC sheets.

(b) Solvent system 95% Ethanol.

(c) Reaction mixture contained 2.5 mCi Na^{125}I.

(d) Reaction mixture was passed through the Dowex column twice to get satisfactory radiochemical purity.

Table 13

^{125}I-Bleomycin A_2 Reaction Mixture After Removal of Free Na^{125}I Using Dowex 1x4 Anion Exchange Resin[(a)]

Reaction	Eastman #6061 System[(b)]			Gelman ITLC System[(a)]		
	Total CPM on Chromatogram	CPM at Na^{125}I Peak	% Free Na^{125}I	Total CPM on Chromatogram	CPM at Na^{125}I Peak	% Free Na^{125}I
1	231212	11480	4.9	233474	10816	4.6
2	223892	9816	4.4	223108	8847	3.9
3	237706	11705	4.9	234195	11274	4.8
4	242729	11008	4.5	240618	10644	4.4

[(a)] 10 ul of Dowex eluate was diluted to 1.0 ml and 10 ul was chromatogrammed on Eastman #6061 strips and in triplicate on Gelman ITLC sheets.

[(b)] 95% ethanol used as solvent system.

b. Chloroform Extraction of ^{125}I-Bleomycin A_2 Reaction Mixture

A chloroform extraction procedure was tried in an effort to remove free Na^{125}I from the ^{125}I-bleomycin A_2 reaction mixture. A 1.4 ml reaction mixture, initially containing trace (20 uCi) quantities of Na^{125}I, was extracted with 2 successive 1.5 ml volumes of chloroform for 15 minutes each. The mixture was stirred continuously throughout the extraction then the chloroform layer was aspirated, collected and assayed for radioactivity. The chloroform extraction procedure was not successful in reducing the concentration of free ^{125}I in the reaction mixture to an acceptable level. The results of 2 extraction experiments is shown in table 14.

While the $CHCl_3$ was found to be unsatisfactory, as an extracting solvent, it is felt that this approach warrants further investigation because it could ultimately provide a means of extracting the radiochemical, and some of the chemical impurities, from the reaction mixture without loss of reaction volume. However, in this study Dowex 1x4 was found to give adequate results and so the solvent extraction procedure was not investigated further.

c. Purification of ^{125}I-Bleomycin A_2 Reaction Mixtures Using Amberlite IRC-50 Cation Exchange Resin

Bleomycin A_2, and presumably ^{125}I-bleomycin A_2, are cations at neutral and acidic pH. Therefore, a cation resin could be used to adsorb ^{125}I-bleomycin A_2 from a reaction mixture leaving impurities, such as Na^{125}I in the supernatant.

Amberlite IR-120, a strongly acidic cation resin, was initially investigated. It was found to take up the ^{125}I-bleomycin A_2 rapidly

Table 14

Extraction of ^{125}I-Bleomycin A_2 Reaction Mixtures with $CHCl_3$

			Extraction Number		
			1	2	
Experiment Number	Total CPM on Chromatogram(a)	CPM in Na^{125}I Peak(a)	CPM in Na^{125}I Peak(b)	CPM in Na^{125}I Peak(b)	Residual Na^{125}I in aqueous phase (%)(a)
1	23512	5122	3719	2643	11.2
2	24908	4619	2919	2112	8.5

(a) Reaction mixture chromatographed on Eastman #6061 silica gel using 95% ethanol as solvent system

(b) $CHCl_3$ layer chromatographed, as in (a).

(c) Aqueous layer chromatographed, as in (a).

from a diluted (1:15) reaction mixture. When the Amberlite IR-120 resin, containing the adsorbed ^{125}I-bleomycin A_2, was separated from the supernatant, 66% of the total radioactivity was found to be retained on the resin. The supernatant was assayed on Eastman silica gel #6061 strips, as previously described, and 84% of the total radioactivity in this fraction was found at Rf 0.80-0.90, the free ^{125}I peak, indicating that the cation resin could be used to separate the ^{125}I-bleomycin A_2 from the unattached Na^{125}I. However, ^{125}I-bleomycin A_2 could not be eluted from the resin.

Amberlite IRC-50, a weakly acidic cation exchange resin was investigated as an alternative. The ^{125}I-bleomycin A_2 reaction mixture was diluted to 15 ml with water and then extracted with 2 grams of Amberlite IRC-50 for 1 hour. When the IRC-50 resin, containing the adsorbed ^{125}I-bleomycin A_2, was separated from the supernatant, 62% of the total radioactivity in the reaction mixture was associated with the resin. The supernatant was assayed on Eastman silica gel chromatograms, as previously described, and 51% of the radioactivity present in the supernatant was found to be unattached ^{125}I.

Extraction of the ^{125}I-bleomycin A_2 from the cation resin was difficult and required continuous elution with methanol. The radioactivity was recovered in 35-40 ml of eluate which was then evaporated to dryness, washed with acetone, dried and then the residue was taken up in aqueous buffer. Less than 3% free Na^{125}I was found in this extracted ^{125}I-bleomycin A_2 fraction, however the procedure required 2-3 hours to complete. It was therefore found to be unsatisfactory and was not investigated further.

d. Purification of ^{125}I-Bleomycin A_2 Reaction Mixtures Using Sephadex G-10 Column Chromatography

Sephadex G-10 column chromatography was discussed previously and while good resolution of the ^{125}I-bleomycin A_2 and Na^{125}I peaks was obtained, variable, but sometimes significant, adsorption of radioactivity occurred in the column. When 6 cm (minicolumns) were used, the ^{125}I-bleomycin A_2 peak was diluted to an unacceptable degree and also contained 10-15% residual Na^{125}I. When 15 cm columns and slower flow rates were used the processing time and adsorption of radioactivity onto the column increased.

A thin 2-4 mm layer of Dowex was packed under 6 cm of sephadex G-10 and while this decreased the amount of free Na^{125}I by about 50% it was impossible to recharge the Dowex resin after use. In spite of the generally unsatisfactory results obtained with Sephadex G-10 it is felt that with a more sophisticated column system and a method of blocking column adsorption, sephadex G-10 could prove to be a method for reducing radiochemical, but not chemical impurities, in ^{125}I-bleomycin A_2 to an acceptable level.

B. Stability of ^{125}I-Bleomycin A_2

1. Stability of ^{125}I-Bleomycin A_2 to Temperature

Following their preparation, solutions of ^{125}I-bleomycin complex and ^{125}I-bleomycin A_2 were assayed chromatographically, at daily intervals for 4 days, using Eastman #6061 silica gel strips and 95% ethanol as solvent system. The radioactivity associated with the ^{125}I-bleomycin A_2 peak at the origin was expressed as a percentage of the total radioactivity present on the chromatogram. A comparison of daily results indicated that there was essentially no difference in the extent or rate of loss of radioactive iodine from the iodinated complex and the iodinated bleomycin A_2. Therefore, it was decided to compare only the stabilities of ^{125}I-bleomycin A_2 produced by the chloramine-T and iodine monochloride methods.

In order to make the results comparable, the initial radioactivity in the ^{125}I-bleomycin A_2 fraction, in the first day of the study, was assumed to be 100% and subsequent similarly determined values for ^{125}I-bleomycin A_2, corrected for physical decay, were expressed as a percent of this initial value.

The amount of radioactivity retained at the ^{125}I-bleomycin A_2 peak when stored at 4°C and 20°C is shown on table 15 and Appendix 3. The loss of radioactivity from the bleomycin is about 1% per day when solutions are stored at 20°C and about 0.7% per day when stored at 4°C.

Fifty ul aliquots of the solutions being compared were chromatographed on silica gel TLC plates using 10% NH_4OOCCH_3:CH_3OH(1:1) as solvent system, on days 1, 5, 10 and 30 of the test period. The chromatograms were visualized under UV light and the position of the bleomycin A_2 was confirmed at Rf 0.35. No other spots were observable on the chromatogram. The plates were marked off in 1 cm segments and then each segment was assayed

Table 15

Stability of ^{125}I-Bleomycin A_2, Produced by ICl Method at 4°C and 20°C[a]

Percent Label Remaining[b]

Day	4°C	20°C
1	100.0	100.0
2	97.2±0.7	96.1±4.0
3	95.8±1.9	95.0±3.1
4	94.3±1.7	94.2±2.9
5	93.4±1.2	93.2±2.3
6	92.9±1.2	93.0±2.0
7	92.1±1.0	91.8±2.5
8	91.4±1.1	92.2±3.3
9	89.8±1.3	90.6±3.9
10	87.9±0.9	90.7±4.4
20	79.1±2.9	86.2±3.1
30	69.7±0.4	79.9±5.0
Total label lost	30.3	20.1
Label lost/day	1.0±0.0%	0.7±0.1%

[a] Assays carried out on Eastman #6061 using 95% ethanol as solvent. All values were corrected for background and physical decay.

[b] Expressed as mean of 3 values ± standard deviation.

individually for radioactivity. The ^{125}I-bleomycin A_2 radioactivity coincided with the observed bleomycin A_2 spot. No other areas of radioactivity were observed except for the ^{125}I peak which was detected at Rf 0.80-0.90.

Therefore, it appeared that ^{125}I-bleomycin A_2 dehalogenated without undergoing any molecular fragmentation that could be detected by chromatographic means. Dehalogenation was probably related to hydrolysis which would occur in aqueous systems. Molecular fragmentation of the iodinated compound, by radiation effects, would not be expected because of the low radioconcentration of the ^{125}I-bleomycin A_2.

In another series of experiments assayed solutions of ^{125}I-bleomycin A_2 were subjected to boiling water bath temperatures for 60 minutes. The solutions were cooled, made up to volume where necessary and then again assessed on the 2 chromatographic systems (silica gel tlc and 10% $NH_4OOCCH_3:CH_3OH(1:1)$ and Eastman silica gel strips #6061 and 95% ethanol). The percentage of the ^{125}I-bleomycin A_2 indicated by the radioactivity remaining at the origin after heat treatment was compared.

The results obtained are shown in Table 16. About 86% of the initial radioactivity was recovered at the origin following incubation for 1 hour at 100°C. There was also a concomitant increase in radioactivity detected at the iodide peak, however this increase was not as large as the decrease in the radioactivity that occurred at the origin.

Table 16

Stability of ^{125}I-Bleomycin A_2, Produced by the Chloramine-T and the ICl Methods, to 100°C for 1 Hour

	ICl			Chloramine-T		
Experiment	1	2	3	4	5	6
Initial Percent ^{125}I-bleomycin A_2	100.0	100.0	100.0	100.0	100.0	100.0
Percent ^{125}I-bleomycin A_2 remaining after treatment	92	86	88	85	87	87
Percent activity lost from origin	8	14	12	15	13	13
Average	11 ± 3%			14 ± 1%		

The silica gel tlc plates in this experiment were also visualized under uv light and surveyed for evidence of molecular degradation. In all instances a spot at Rf 0.35 indicated the presence of unlabeled bleomycin A_2. No other spots were observed on the chromatogram. The plates were divided into segments and assayed for radioactivity as previously described. Areas of radioactivity were found at Rf 0.35, corresponding to ^{125}I-bleomycin A_2 and at Rf 0.80-9.90, corresponding to Na^{125}I. Some significant radioactivity was detected between ^{125}I-bleomycin A_2 and ^{125}I-fractions. This radioactivity could represent heavy tailing from the ^{125}I peak or possibly some degradation of the ^{125}I-bleomycin A_2. The radioactivity in these segments however was only 3-5 times background levels.

These stability experiments indicate only that the ^{125}I-bleomycin A_2 was thermodynamically stable for the conditions under which it was tested. However, thermodynamic stability does not necessarily imply *in vivo* stability, as has been shown for ^{111}In-bleomycin (11), which is stable to heat but is unstable *in vivo*.

2. Stability of ^{125}I-Bleomycin A_2 in the Presence of Cu^{+2} *In Vitro*

The *in vitro* stability of a labeled compound, under rigidly controlled laboratory conditions, does not always allow inferences to be made about its *in vivo* stability.

Electron acceptors which can form thermodynamically more stable complexes with a ligand, will displace or exclude thermodynamically less favorable electron acceptors. The *in vivo* environment contains many such electron acceptors which could disrupt a labeled compound. Therefore, in addition to testing the strength of the chemical association by heat, the bond should also be challenged by substances which may be present in the blood and which may have more affinity than the incorporated radionuclide for the binding sites on the ligand. For example, ^{111}In-bleomycin is stable to heat (11) but, the chemical bond is broken within 1 hour in the presence of Cu^{+2}. *In vivo*, ^{111}In-bleomycin molecule was found to be partially disrupted with a significant portion of the ^{111}In being bound to the transferrin in the blood (10)(11). Co-57-bleomycin, however, was found to be stable at elevated temperatures and in the presence of Cu^{+2} (20).

Cu^{+2} ions, form stable chelates with bleomycin (1). Therefore cations, like $^{111}In^{+3}$, which form thermodynamically less favorable complexes, will be displaced from their binding sites on bleomycin. The affinity of $^{57}Co^{+2}$ for binding sites on bleomycin is about the same or stronger than that of Cu^{+2} ions and hence ^{57}Co complexes will be stable.

Iodine is the most electropositive member of the halogen family. It is theoretically capable of forming weak chelate bonds with some ligands (35)(38)(42)(74). Cu^{+2} should therefore readily displace iodine from its binding sites on bleomycin if the association was only due to a weak chelation effect. However, the presence of Cu^{+2} would be expected to have no effect on the stability of a covalent iodine-bleomycin association because of the basic difference in the two bond types. At neutral pH Cu^{+2} would have strong affinity for such ready electron donors as the nitrogens on imidazole and pyrimidine or the sulfur and nitrogen atoms on the thiazole rings. Iodine, in contrast would show only weak affinity for these sites(40)(41) in the bleomycin molecule preferring instead a site such as the C-2 position on the imidazole ring of the B-hydroxyhistidine moiety, as has been suggested (58).

To test this hypothesis, a solution of ^{125}I-bleomycin A_2 was chromatographed on silica gel tlc plates and assayed for radiochemical purity on Eastman silica gel #6061 strips. Then an equal volume of $CuCl_2$ solution (10 ug/ul of Cu^{+2}) was added to the ^{125}I-bleomycin A_2 solution and incubation was allowed to proceed for 30 minutes. The solution was again chromatographed on silica gel tlc and assayed for radiochemical purity on Eastman #6061 strips.

When the silica gel tlc plates were visualized using uv light, the bleomycin A_2 fraction was detected at Rf 0.35 with no other spots being observed. The silica gel plates were then marked off into 1 cm segments and assayed for radioactivity as previously described. Radioactivity was detected at Rf 0.35, indicating only the presence of ^{125}I-bleomycin A_2. The region between Rf 0.40-0.70 contained some significant radioactivity, about 8-10 times background level in each segment. Radioactivity was not detected at Rf 0.70-0.90 indicating that free Na^{125}I was not present in the solution.

Aliquots of the reaction mixture were chromatographed on Eastman #6061 silica gel using 95% ethanol as solvent. The chromatogram was divided into 1 cm segments and each segment was assayed individually for radioactivity. The quantity of radioactivity detected at the origin indicated that ^{125}I-bleomycin A_2 was stable in the presence of Cu^{+2}. Only background radioactivity was detected in the region Rf 0.75-0.90 indicating that free Na^{125}I was not present in the solution. Significant radioactivity, 5-6 times background, was detected in the region Rf 0.30-0.40.

The radioactivity detected at an Rf 0.30-0.40 was considered to be another molecular species in the reaction mixture that was less polar than ^{125}I-bleomycin A_2. This unknown moiety was binding free $Na^{125}I$ that was present in the solution. The nature of this unknown species was not investigated further. However, it is known that Cu^{+2} is stabilized by imidazole in the presence of iodide on the basis of considerations involving redox potentials (36). Although various associations of Cu-ligand-iodide are known, a definitive discussion of these would be outside the realm of this study. A recent review of this topic is presented by Sundberg (36).

C. Chemical Purity of ^{125}I-Bleomycin A_2 Solutions

The principal chemical impurity present in ^{125}I-bleomycin A_2 solution may be considered to be unlabeled bleomycin A_2. The only way in which this chemical impurity can be reduced is by decreasing the quantity of bleomycin A_2 and increasing the quantity of $Na^{125}I$ that is used in the reaction mixture.

In the present study it was felt that 150 ug of bleomycin A_2 would be the smallest practical quantity that could be used because bleomycin, being very polar, was strongly adsorbed onto glassware and millipore filters (20) and losses in dilute solution would be excessive. On the other hand, in order to obtain a 1:1 molar ratio between $Na^{125}I$ and bleomycin A_2 (assuming a 100% incorporation of ^{125}I into one iodination site per molecule) would require 225 mCi of $Na^{125}I$ or 1630 mCi of $Na^{131}I$. The calculation of these theoretical values is shown in Appendix 4.

In the actual experimental reaction mixtures, 150 ug of bleomycin A_2 was reacted with 2.5 mCi of $Na^{125}I$ and 80% of the iodine was incorporated into the bleomycin A_2. This gave $8.9x10^{-3}$

atoms of ^{125}I per molecule of bleomycin A_2 or a specific activity of 13.3 uCi/ug. The calculations of these results is shown in Appendix 5.

^{125}I-bleomycin A_2 solutions, for biological studies, were prepared using routinely accepted aseptic and sterile techniques. The final solutions were passed through 0.22 micron millipore filters into sterile pyrogen-free vials. The quantity of radioactivity retained on filters was estimated to be 40-45% of the total radioactivity in the solution. Thus sterilization by millipore filtration results in very significant loss of radioactivity. However, ^{125}I-bleomycin, being heat stable could possibly be sterilized by terminal autoclaving.

D. Nuclear Magnetic Resonance (NMR) Studies on ^{125}I-Bleomycin A_2

The bleomycin molecule contains, as one of its structural components, a B-hydroxyhistidyl moiety. Based on theoretical and experimental considerations (40)(41)(58)(70)(71)(76) the most likely site for the covalent incorporation of iodine into bleomycin would be at the C-2 position on the imidazole ring of the B-hydroxyhistidyl residue. At this position, the carbon-iodine bond would be stable to heat and to treatment with reducing agents (40)(41). According to Umezawa, et al. (22)(23)(24) the C-2 proton of the imidazole ring would appear in the nmr spectrum as a doublet (J = 1.5 cps)(a) in the region of 8.16 ppm(b) when deuterated methanol was used as the solvent system and tetramethylsilane (tms) as the internal reference standard.

(a) J is a coupling constant that is characteristic of the distance in cycles per second (cps) between the individual peaks of the doublet signal.

(b) P.P.M. is parts per million and is a measure of the chemical shift of a proton.

In an attempt to determine if the site of iodination in bleomycin was at the C-2 position of the imidazole ring, a 0.001 M bleomycin A_2 working standard (National Institutes of Health, Tokyo, Japan) was dissolved in deuterated methanol and subjected to multiple scans using a 100 megahertz A100D Analytical NMR spectrometer. (Varian Associates). The instrument was not sensitive enough to satisfactorily resolve the doublet signal at 8.16 ppm although a doublet signal was somewhat distinguishable in this region.

An identical solution of bleomycin A_2 working standard was iodinated using ICl as the source of non-radioactive iodine and the iodinated bleomycin was extracted as previously described using Amberlite (IRC-50 weakly acidic cation exchange resin. The iodinated bleomycin was extracted from the resin using methanol which was then evaporated to dryness. The residue was taken up in ethanol and recrystallized as previously described. The crystals were dissolved in deuterated methanol and subjected to multiple nmr scans as before. The vaguely discernible doublet peak at 8.16 ppm in the bleomycin A_2 scans was not observed in the iodinated bleomycin A_2 scans indicating that the proton at C-2 was being replaced by the iodine atom. The resolution of the A100D Analytic NMR spectrometer was not good enough to provide clear-cut data and it was felt that further investigation of the nmr spectrum of iodinated bleomycin was warranted using the more sensitive A200 series.

E. Radiochemical Purity of ^{114m}In-Bleomycin A_2

The radiochemical purity of ^{114m}In-bleomycin as prepared for animal distribution studies was assessed on silica gel tlc plates and Eastman #6061 silica gel strips using 10% NH_4OOCCH_3:CH_3OH(1:1) as the solvent systems. When silica gel tlc plates were visualized under uv light bleomycin A_2 was detected at Rf 0.35. The presence of other compounds were not detected. The silica gel plate was divided into 1 cm segments and individually assessed for radioactivity in an appropriately calibrated gamma counter (Beckman Biogamma, Beckman Instruments Inc., Fullerton, Calif.). Radioactivity was detected at the origin, indicating presence of $^{114m}In(OH)_3$ and at Rf 0.35, indicating the presence of ^{114m}In-bleomycin A_2. No other areas of radioactivity were detected.

When the ^{114m}In-bleomycin A_2 reaction mixture was assayed on Eastman silica gel #6061 strips using 10% NH_4OOCCH_3:CH_3OH(1:1) as solvent system areas of radioactivity were detected at the origin and in a band between Rf 0.50-0.60. The amount of radioactivity detected at the origin was 6.8% of the total radioactivity on the chromatogram whereas 92.5% of the radioactivity was associated with the region at Rf 0.50-0.60. No other areas of radioactivity were detected. The ^{114m}In-bleomycin A_2 was therefore 92.5% radiochemically pure and was used, as described, without further purification.

V. Tissue Distribution Studies

A. Preliminary Tissue Distribution in Mice

A preliminary tissue distribution study of ^{125}I-bleomycin complex solution was carried out using groups of 3 tumor-bearing mice at intervals of 1.5, 3.0 and 6.0 hours after intravenous injection. A wide selection of tissues were excised, blotted free of blood and assayed, in glass counting vials, in a gamma counter (Nuclear Chicago 1125, Searle Analytic). The preliminary results were tabulated in cpm/mg of tissue and are shown in Appendix 6. The results indicated that significant radioactivity was present in the lungs, liver, testes, kidneys, spleen, muscle, tumor, blood, skin and gut while lesser amounts were detected in the brain and bone. The radioactivity in the bladder and heart were largely attributed to radioactivity in the urine and blood respectively. It was felt from this preliminary study that lung, liver, kidney, spleen, testes, blood, tumor and muscle represented the organs of interest with regard to uptake of radioiodinated bleomycin. The carcass was divided into trunk, tail, and head and each portion was assayed for radioactivity in a small animal whole body counter consisting of 2 shielded 3"x3" NaI(Tl) crystals. The results obtained are shown in Appendix 6. There was no unusual accumulation of radioactivity in the head, which would occur if the iodine-bleomycin was unstable *in vivo*, with resultant uptake of free radioiodide by the thyroid gland. Analysis of the tail for residual radioactivity was done to ensure that significant amounts of injected material was not retained at the injection site.

B. Tissue Distribution of ^{125}I-Bleomycin Complex in Normal Mice

Tissue distribution studies were done using 20-25 gram adult male Swiss mice at various time intervals after the intravenous administration of 27 uCi of ^{125}I-bleomycin complex. The ^{125}I-bleomycin complex was produced by the ICl method and contained 27 uCi in 0.1 ml of solution at a specific activity of 13.3 mCi/mg. Groups of 6 mice were sacrificed, by cervical dislocation, at 1, 2, 3, 4, 6, 12 and 24 hour time intervals. Blood was immediately removed from the heart and placed in a glass counting vial. The designated tissues and organs were removed, blotted free of blood, weighed and placed in glass counting vials. The radioactivity in each sample was determined on an appropriately calibrated gamma counter (Nuclear Chicago 1125, Searle Analytic). The results are shown in Table 17 and Fig. 9.

Blood, kidney and testes showed significant uptake of radioactivity at one hour after i.v. administration of ^{125}I-bleomycin complex. Radioactivity was rapidly cleared from all tissues except the kidney which had high levels that persisted throughout the period of the study.

C. Tissue Distribution of ^{125}I-Bleomycin A_2 in Normal Mice

The tissue distribution study just described was repeated except that 25 uCi of ^{125}I-bleomycin A_2 instead of the ^{125}I-bleomycin complex was administered to normal mice. The bleomycin A_2 was isolated from the commercially available bleomycin complex and purified using IRC-50 resin, as previously described. The bleomycin A_2 was then iodinated by the ICl method to a specific activity of

Table 17

Tissue Uptake in Normal Mice After the Intravenous Administration of ^{125}I-Bleomycin Complex[(a)(b)(c)]

	Time (Hours) After Administration				
Tissue	T1	T2	T3	T4	T6
lung	0.45±0.04	0.22±0.04	0.21±0.03	0.05±0.01	0.02±0.00
liver	0.19±0.04	0.11±0.03	0.06±0.01	0.04±0.01	0.03±0.01
testes	0.47±0.17	0.41±0.06	0.34±0.04	0.11±0.01	0.02±0.01
spleen	0.10±0.05	0.03±0.00	0.03±0.01	0.02±0.01	0.02±0.00
kidney	2.10±0.41	0.98±0.18	0.57±0.07	0.50±0.05	0.41±0.04
muscle	0.23±0.04	0.11±0.01	0.06±0.01	0.03±0.01	0.01±0.01
blood(d)	1.29±0.11	0.83±0.06	0.13±0.02	0.06±0.01	0.02±0.00

(a) Expressed as percent dose per gram of tissue. Mean of 6 animals ± standard deviation.

(b) ^{125}I-bleomycin complex was produced by the ICl method.

(c) Each animal was injected with 0.1 ml ^{125}I-bleomycin complex (27 uCi).

(d) Percent of injected activity per 0.1 ml of whole blood. Mean of 6 animals ± standard deviation.

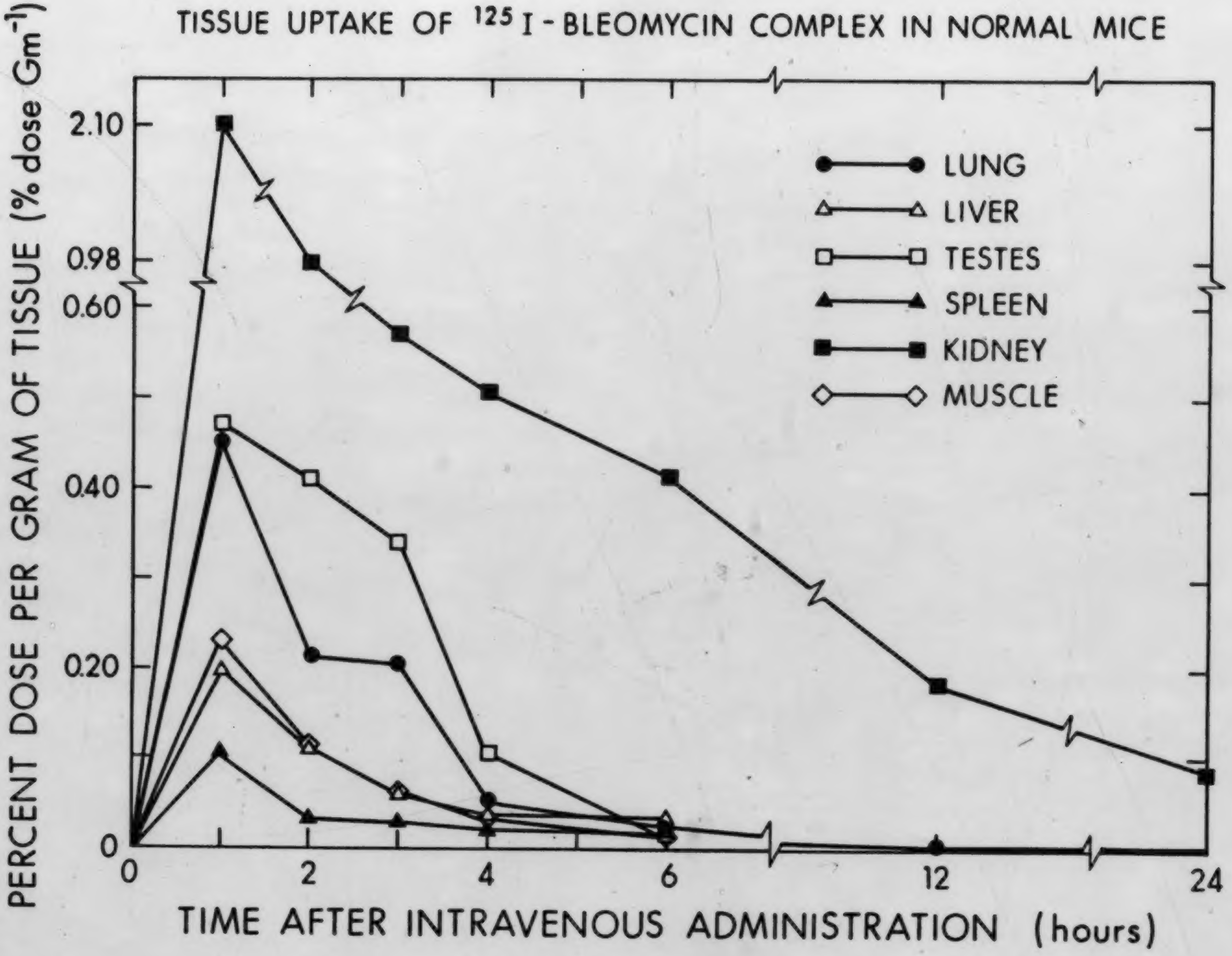
TISSUE UPTAKE OF ^{125}I - BLEOMYCIN COMPLEX IN NORMAL MICE
PERCENT DOSE PER GRAM OF TISSUE (% dose Gm^{-1})
2.10
0.98
0.60
0.40
0.20
0
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)
0
2
4
6
12
24
LUNG
LIVER
TESTES
SPLEEN
KIDNEY
MUSCLE

Figure 9

13.1 mCi/mg. After the intravenous administration of 25 uCi of ^{125}I-bleomycin A_2 in 0.1 ml of solution, groups of 6 mice were sacrificed at the designated time intervals and their tissues were excised and assayed for radioactivity. The results are shown in Table 18 and Figure 10.

Blood, kidney and testes showed significant uptake of radioactivity at one hour after i.v. administration of bleomycin A_2. Radioactivity was rapidly cleared from all tissues except the kidney. The tissue distributions of ^{125}I-bleomycin A_2 and ^{125}I-bleomycin complex were found to be similar in all respects.

D. Tissue Distribution of ^{125}I-Bleomycin Complex in Tumor-Bearing Mice

Tissue distribution studies were done using Swiss mice bearing solid Ehrlich's ascites tumor implanted in their right femoral region. Six mice were sacrificed at each of the designated time intervals after the intravenous administration of 20 uCi of ^{125}I-bleomycin complex which was produced by the ICl method to a specific activity of 14.0 mCi/mg. The tissues were excised and assayed for radioactivity. The results of this tissue distribution study are shown in Table 19 and Figures 11 and 12. High uptake of radioactivity was observed for tumor, which at 6 hours gave maximum tumor:muscle and tumor:blood ratios of 16:1 and 8:1 respectively as summarized in Table 20. However, the absolute concentrations of radioactivity in the tissues at after 6 hours was found to be relatively low. Initially, high levels of radioactive uptake

Table 18

Tissue Uptake in Normal Mice After the Intravenous Administration of ^{125}I-Bleomycin A_2 (a)(b)(c)

Tissue	Time (Hours) After Administration				
	T1	T2	T3	T4	T6
lung	0.48±0.05	0.22±0.04	0.21±0.04	0.06±0.01	0.02±0.00
liver	0.20±0.05	0.11±0.02	0.06±0.01	0.04±	0.03±0.01
testes	0.51±0.12	0.41±0.09	0.35±0.04	0.10±0.00	0.02±0.00
spleen	0.16±0.03	0.03±0.01	0.03±0.01	0.03±0.00	0.02±0.00
kidney	2.10±0.32	0.95±0.15	0.59±0.06	0.50±0.09	0.43±0.04
muscle	0.23±0.04	0.10±0.01	0.06±0.02	0.04±0.02	0.01±0.00
blood(c)	1.30±0.05	0.83±0.06	0.11±0.02	0.05±0.01	0.02±0.00

(a) Expressed as percent dose per gram of tissue. Mean of 6 animals ± standard deviation.

(b) Each animal was injected with 0.1 ml ^{125}I-bleomycin A_2 solution (25 uCi).

(c) Bleomycin A_2 was isolated from the complex (Bleonane, Bristol Labs.) and iodinated using the ICl method.

(d) Expressed as percent dose per 0.1 ml of whole blood, mean of 6 animals ± standard deviation.

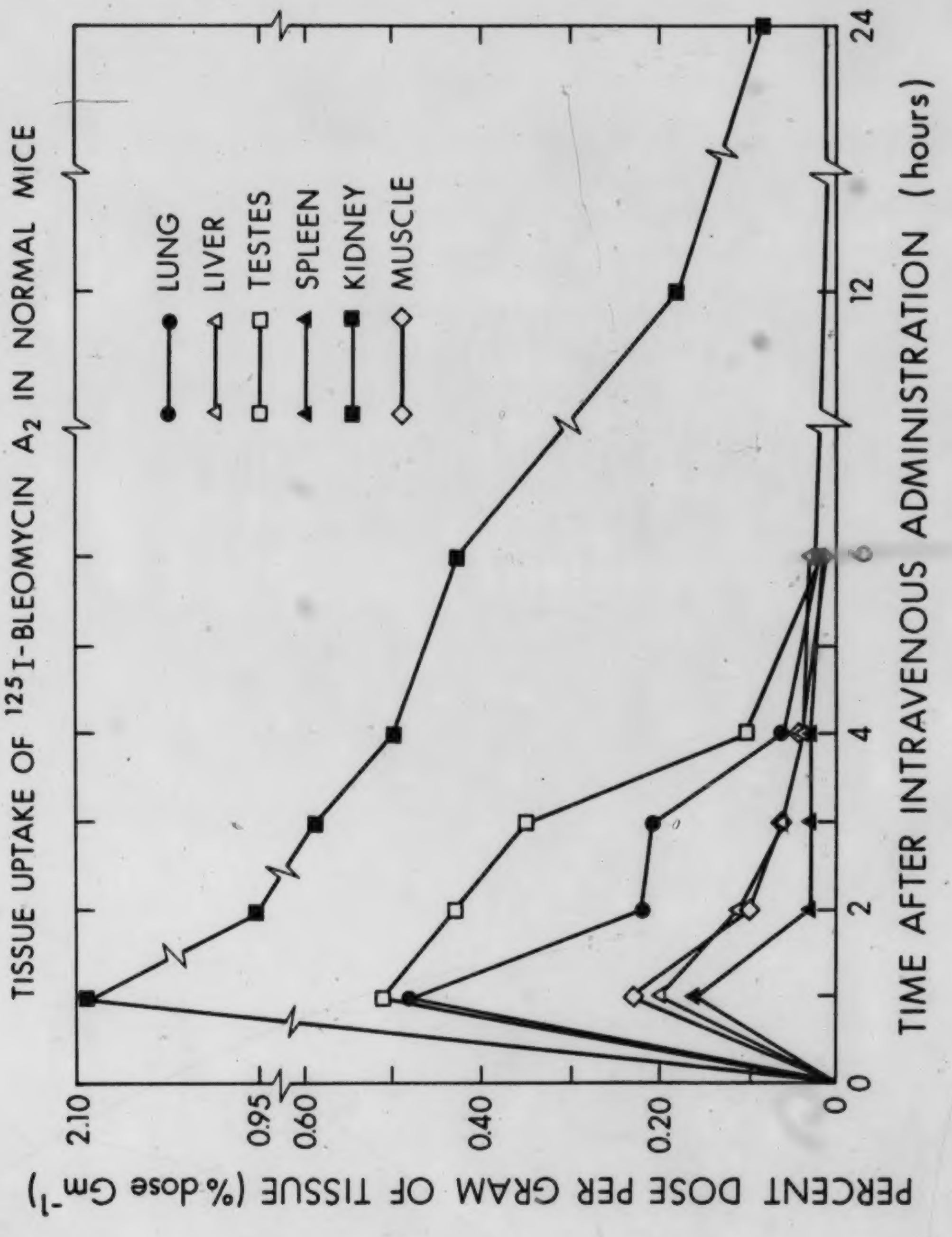
TISSUE UPTAKE OF ^{125}I-BLEOMYCIN A$_2$ IN NORMAL MICE
LUNG
LIVER
TESTES
SPLEEN
KIDNEY
MUSCLE
PERCENT DOSE PER GRAM OF TISSUE (% dose Gm^{-1})
2.10
0.95
0.60
0.40
0.20
0
0
2
4
6
12
24
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)

Figure 10

Table 19

Tissue Uptake in Tumor-Bearing Mice After the Intravenous Administration of ^{125}I-Bleomycin Complex(a)(b)(c)

Tissue	Time (Hours) After Administration				
	T1	T2	T3	T4	T6
lung	0.46±0.04	0.19±0.06	0.22±0.03	0.05±0.01	0.02±0.00
liver	0.21±0.07	0.11±0.02	0.05±0.01	0.05±0.01	0.03±0.01
testes	0.51±0.22	0.42±0.07	0.35±0.06	0.08±0.03	0.02±0.01
spleen	0.18±0.04	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01
kidney	2.03±0.35	0.87±0.21	0.57±0.07	0.48±0.09	0.44±0.06
muscle	0.22±0.03	0.10±0.01	0.06±0.02	0.03±0.01	0.01±0.00
tumor	0.55±0.05	0.52±0.03	0.48±0.05	0.30±0.04	0.16±0.03
blood(d)	1.27±0.06	0.79±0.08	0.13±0.01	0.06±0.01	0.02±0.00

(a) Expressed as percent dose per gram of tissue. Mean of 6 animals ± standard deviation.

(b) Each animal was injected with 0.1 ml ^{125}I-bleomycin complex (24 uCi).

(c) Blenoxane, Bristol Labs.

(d) Expressed as percent dose per 0.1 ml of whole blood. Mean of 6 animals ± standard deviation.

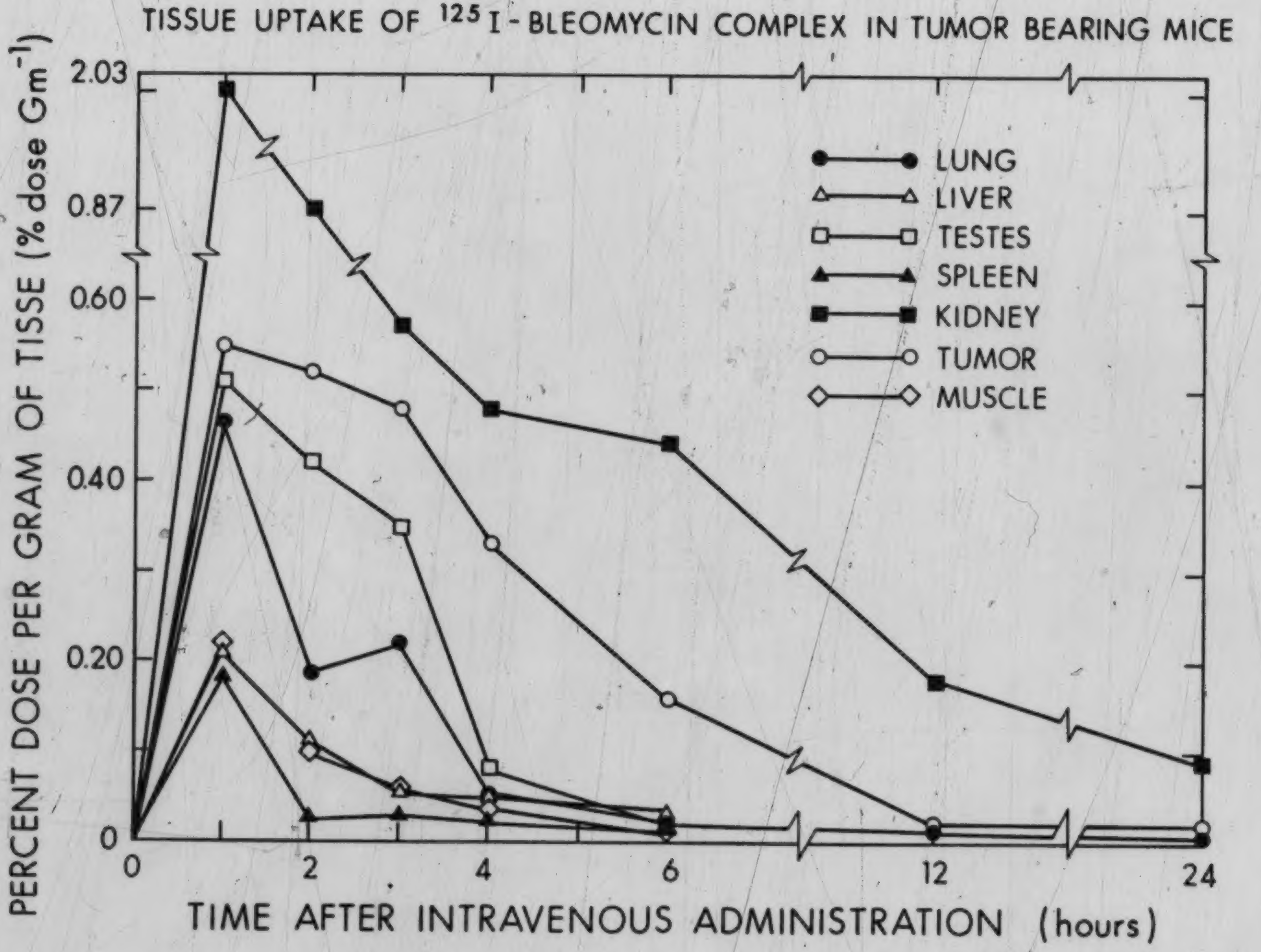
TISSUE UPTAKE OF ^{125}I - BLEOMYCIN COMPLEX IN TUMOR BEARING MICE
PERCENT DOSE PER GRAM OF TISSE (% dose Gm^{-1})
2.03
0.87
0.60
0.40
0.20
0
0
2
4
6
12
24
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)
● LUNG
△ LIVER
□ TESTES
▲ SPLEEN
■ KIDNEY
○ TUMOR
◇ MUSCLE

Figure 11

UPTAKE IN MUSCLE, TUMOR, TESTES AND BLOOD OF ^{125}I - BLEOMYCIN COMPLEX IN TUMOR - BEARING MICE

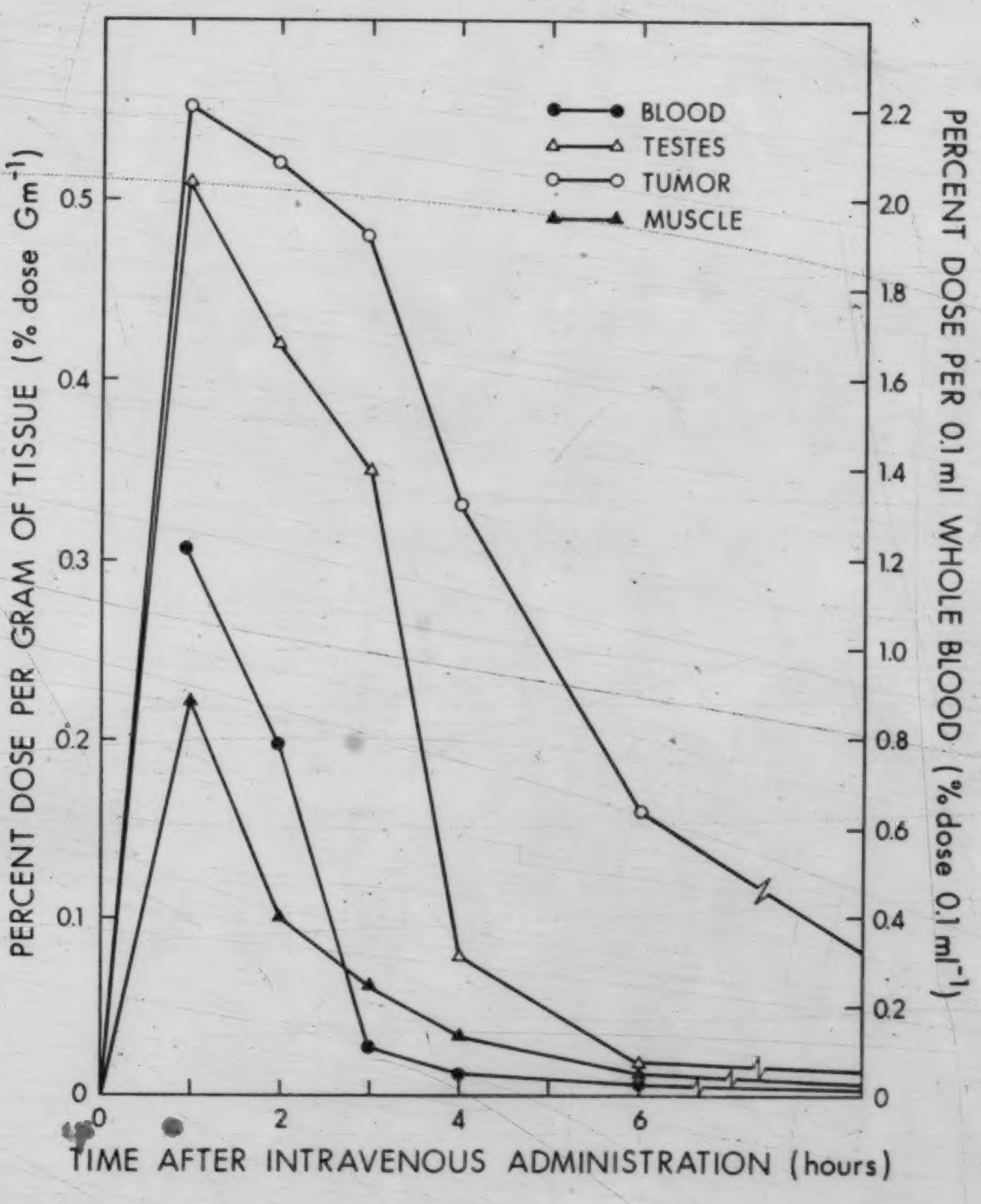

Figure 12

Table 20

Tumor:Muscle and Tumor:Blood Ratios[a][b][c] Obtained for ^{125}I-Bleomycin Complex in Tumor-Bearing Mice

Ratio	Time (Hours) After Administration				
	T1	T2	T3	T4	T6
Tumor:Muscle	2.5	5.2	8.0	10.0	16.0
Tumor:Blood	0.43	0.66	3.7	5.0	8.0

(a) Comparison of percent injected dose per gram of tumor tissue to the percent of injected dose per gram of muscle or 0.1 ml of whole blood.

(b) Expressed as a mean ± standard deviation of 6 mice at each time interval.

(c) Radioactivity in tissues at 12 and 24 hours after injection was extremely low.

were observed in the lung, kidney, tumor, testes and blood with levels falling rapidly 2 hours after injection in all tissues except tumor and kidney. The data from these experiments is comparable with a study using the same mouse tumor model and ^{57}Co-bleomycin which showed a similar pattern of radioactive uptake and clearance (9). ^{57}Co-bleomycin radioactivity was rapidly cleared from all tissues except tumor with maximum tumor:muscle ratio of 24:1 being reported at 24 hours after administration (9). This study also used ^{57}Co-bleomycin solutions labeled to different specific activities. The low specific activity preparation had a tumor:muscle ratio of only 12:1 compared to 24:1 reported for the higher specific activity preparation (9).

In contrast, tissue distribution studies using ^{111}In-bleomycin in mice bearing a variety of tumors indicated that maximum tumor:muscle and tumor:blood ratios of about 6:1 and 9:1 respectively occurred at 48 hours after injection (21). Levels of ^{111}In-bleomycin radioactivity remained relatively high in all tissues studied with accumulation of radioactivity occurring in liver and spleen.

In a study involving tumor (sarcoma)-bearing mice (20), Tc-bleomycin was found to be cleared rapidly from all tissues except tumor and kidney. Radioactivity in tumor remained at elevated levels even after 24 hours, with maximum tumor:blood ratios of 18:1 being observed at this time. The absolute concentrations of radioactivity however at 24 hours was found to be very small and an optimum tumor:blood ratio of 11:1 was found at 6 hours after administration (20). Clearance of radioactivity from the body was found to be rapid with only 15% of the dose being recovered after 24 hours (20).

Therefore on the basis of similar studies involving a variety of mouse-tumor models it would appear that iodinated bleomycin complex gave a superior tumor:tissue ratio at 6 hours after administration and a comparable or higher tumor:blood ratio than either the indium or technetium labeled bleomycins. Only ^{57}Co-labeled bleomycin had higher tumor:muscle and tumor:blood ratios (9) at all time intervals studied. However the long half-life of Cobalt-57 would preclude any extensive clinical application of this radiopharmaceutical. Thus it is postulated that the use of ^{123}I for radioiodination of bleomycin may yield a clinically useful tumor imaging agent.

E. Tissue Distribution of ^{125}I-Bleomycin A_2 in Tumor-Bearing Mice

Tissue distribution studies were done using 6 tumor-bearing mice at each time interval after intravenous administration of 26 uCi in 0.1 ml of ICl produced ^{125}I-bleomycin A_2 at a specific activity of 12.9 mCi/mg. The solid form of Ehrlich's ascites tumor was allowed to grow 7 days in the right femoral region of 20-25 gram Swiss mice. The results are shown in Table 21 and Figures 13 and 14. Initially, high levels of radioactive uptake were observed in lung, kidney, tumor, testes and blood with levels falling rapidly 2 hours after injection in all tissues except tumor and kidney. Clearance of radioactivity in all tissues appeared to parallel the clearance of radioactivity from the blood, which had

Table 21

Tissue Uptake in Tumor-Bearing Mice After the Intravenous Administration of ^{125}I-Bleomycin A_2 (a)(b)(c)(d)

Tissue	Time (Hours) After Administration				
	T1	T2	T3	T4	T6
Lung	0.47±0.06	0.18±0.04	0.22±0.03	0.05±0.01	0.02±0.00
liver	0.19±0.03	0.11±0.02	0.05±0.00	0.05±0.01	0.03±0.01
testes	0.61±0.25	0.40±0.10	0.34±0.03	0.06±0.03	0.02±0.00
spleen	0.17±0.04	0.03±0.00	0.03±0.00	0.02±0.01	0.02±0.00
kidney	2.17±0.22	0.81±0.15	0.57±0.06	0.47±0.08	0.45±0.09
muscle	0.21±0.02	0.11±0.01	0.06±0.01	0.03±0.01	0.01±0.00
tumor	0.55±0.05	0.52±0.03	0.48±0.03	0.33±0.03	0.16±0.02
blood	1.35±0.08	0.87±0.05	0.13±0.02	0.05±0.01	0.02±0.00

(a) Expressed as percent dose per gram of tissue. Mean of 6 animals ± standard deviation.

(b) Each animal was injected with 0.1 ml ^{125}I-bleomycin A_2 solution (26 uCi).

(c) Bleomycin A_2 was isolated from the complex (Blenoxane, Bristol Labs.) and iodinated using the ICl method.

(d) Expressed as percent dose per 0.1 ml of whole blood. Mean of 6 animals ± standard deviation.

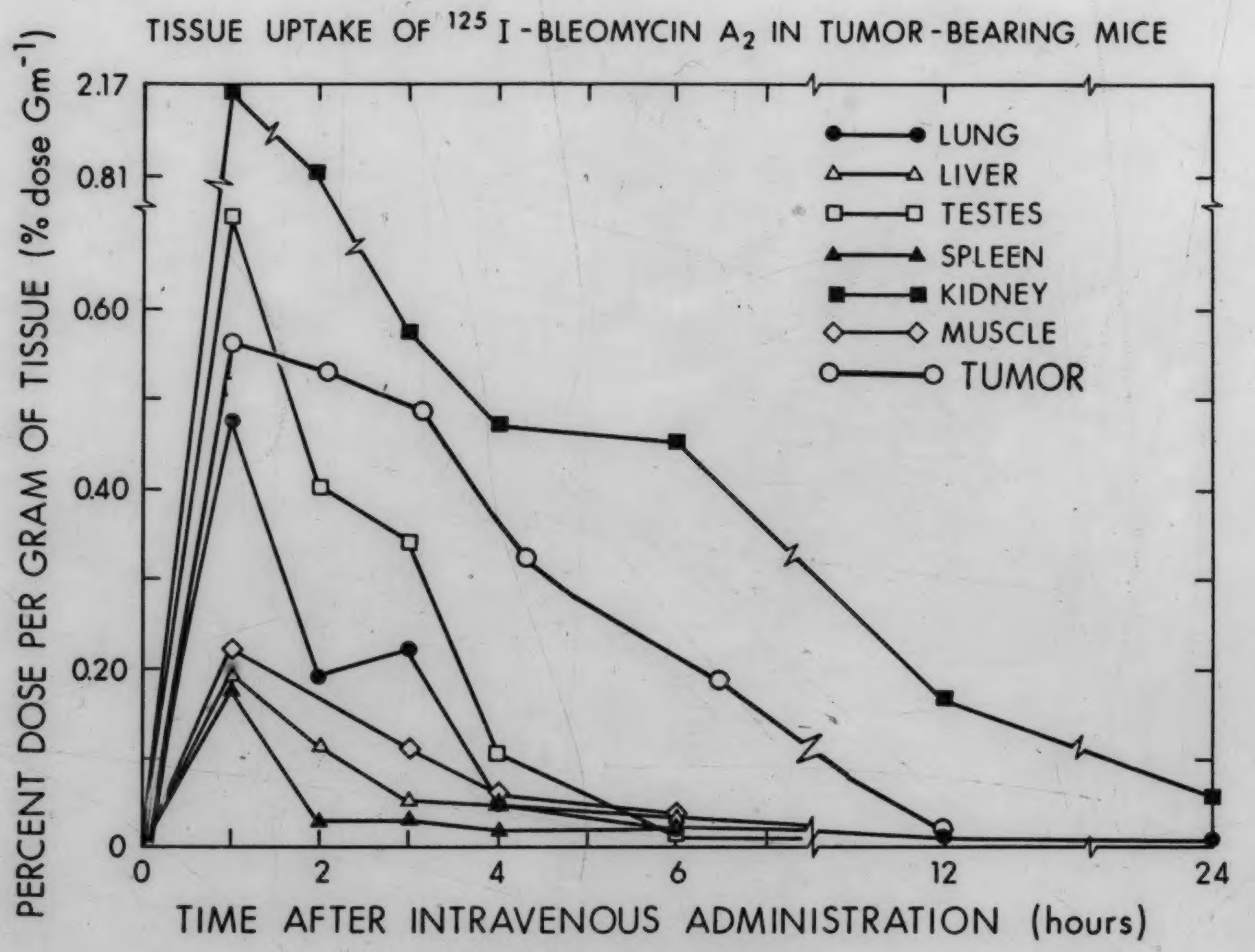
TISSUE UPTAKE OF ^{125}I-BLEOMYCIN A_2 IN TUMOR-BEARING MICE
PERCENT DOSE PER GRAM OF TISSUE (% dose Gm^{-1})
2.17
0.81
0.60
0.40
0.20
0
2
4
6
12
24
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)
LUNG
LIVER
TESTES
SPLEEN
KIDNEY
MUSCLE
TUMOR

Figure 13

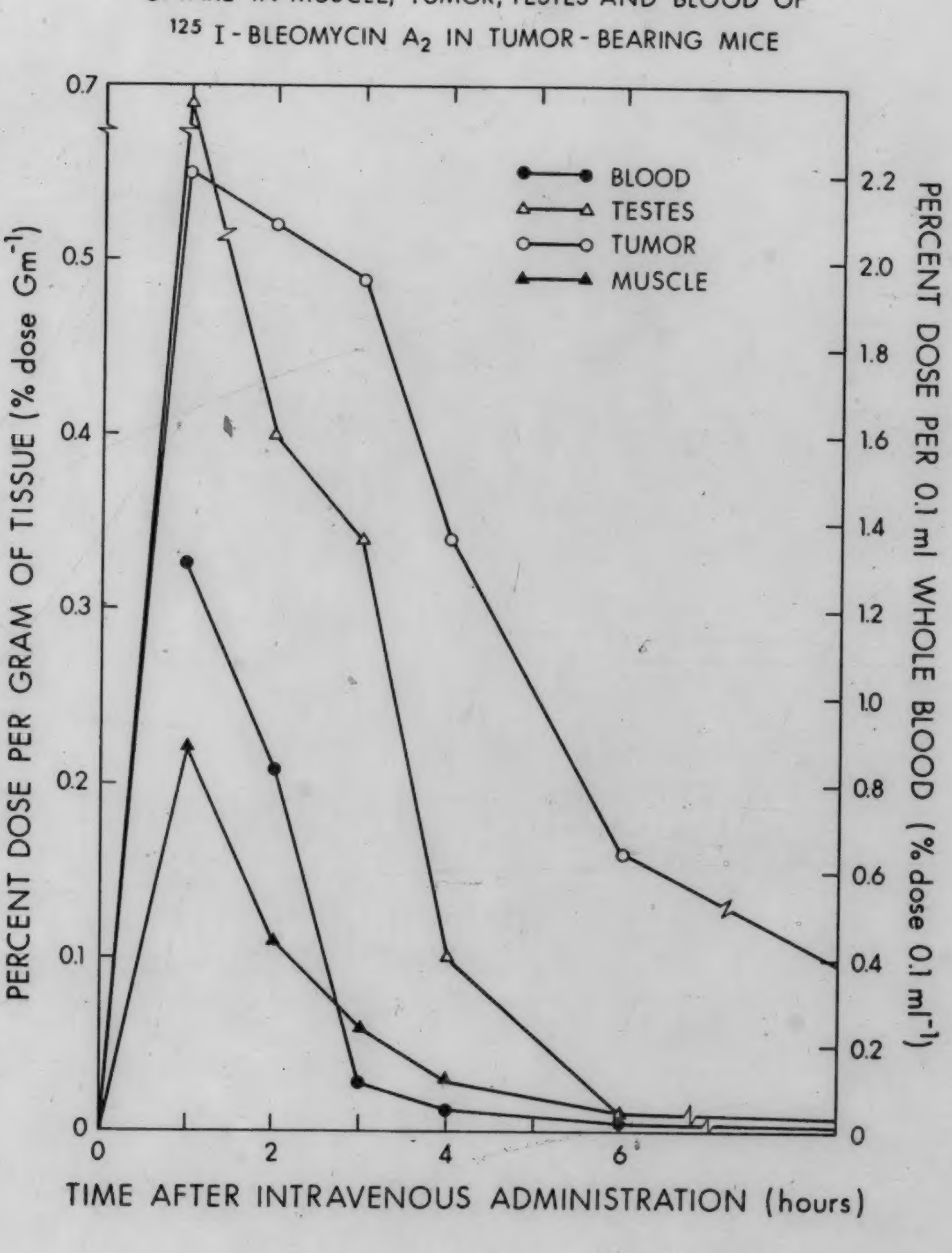
UPTAKE IN MUSCLE, TUMOR, TESTES AND BLOOD OF
^{125}I-BLEOMYCIN A_2 IN TUMOR-BEARING MICE
BLOOD
TESTES
TUMOR
MUSCLE
PERCENT DOSE PER GRAM OF TISSUE (% dose Gm^{-1})
0
0.1
0.2
0.3
0.4
0.5
0.7
PERCENT DOSE PER 0.1 ml WHOLE BLOOD (% dose 0.1 ml^{-1})
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
2
4
6
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)

Figure 14

a 1.25% dose per 0.1 ml at 1 hour falling to 0.1% dose per 0.1 ml at 3 hours after injection. Tumor-to-muscle and tumor-to-blood ratios of 16:1 and 8:1 respectively were observed at 6 hours after administration as shown in Table 22.

There was found to be no significant difference in the tissue distribution of ^{125}I-bleomycin complex and ^{125}I-bleomycin A_2 in normal mice when the results for the 2 sets of data were compared using a t-test at the 5% level of significance and 10 degrees of freedom. A similar comparison of results obtained for tissue distribution studies done in normal and in tumor-bearing mice also indicates that the two radioiodinated substances were not handled in a significantly different manner in vivo. This was not unexpected since bleomycin complex is composed of 45-70% bleomycin A_2 and up to 27% bleomycin B_2.

In a recent study involving tumor-bearing rats (47) the tissue distribution of A_2 and B_2 fractions labeled with cobalt-57 showed higher concentrations in tissues and tumors than other similarly labeled bleomycin fractions. It was also reported that the tissue distributions of A_2 and B_2 fractions were highly comparable and they both gave tissue distributions that were not significantly different from that obtained using the ^{57}Co-bleomycin complex (47).

These results would appear to suggest that there is no real justification for using the iodinated A_2 fraction in preference to the iodinated complex especially when the financial costs of isolating pure A_2 from the bleomycin complex are considered.

Table 22

Tumor:Muscle and Tumor:Blood Ratios[a][b][c] Obtained for ^{125}I-Bleomycin A_2 in Tumor-Bearing Mice

	Time (Hours) After Administration				
Ratio	T1	T2	T3	T4	T6
Tumor:Muscle	2.5	4.7	8.2	11	16
Tumor:Blood	0.43	0.62	4.5	7.3	7.6

[a] Comparison of percent injected dose per gram of tumor tissue to the percent of injected dose per gram of muscle or 0.1 ml of whole blood.

[b] Expressed as a mean ± standard deviation of 6 mice at each time interval.

[c] Radioactivity in tissues at 12 and 24 hours after injection was extremely low.

A comparison of the tissue distribution data for normal and tumor-bearing mice indicates that the iodinated bleomycins are rapidly cleared from tissues and at 6 hours after administration, all tissues, with the exception of blood, kidney and tumor, contained very low levels of radioactivity. This would suggest that the iodinated bleomycins are stable in vivo because the radioactivity in the blood was still considerable, relative to other tissues, at 6 hours after administration at this time. The percent of the injected dose per 0.1 ml of whole blood was 0.02% which represented 0.4% of the total dose contained in all of the blood (assuming that a 25 gm mouse had a blood volume of 2 ml. If dehalogenation had occurred in vivo much of the free ^{125}I would be rapidly trapped by the thyroid and radioactivity in the blood would be relatively low.

The uptake and slow excretion of radioactivity observed for tumor may, in part, be a function of the persistent levels of radioactivity in the blood. It is known, for example, that bleomycin binds to DNA in tumors and in other rapidly growing cells (27). Since iodine, covalently attached to the B-hydroxyhistidyl, probably would not unduly alter the conformation of the bleomycin molecule, the biological behavior of bleomycin and iodinated bleomycin should be comparable. Therefore, iodinated bleomycin would tend to accumulate in tumor as it became attached to the tumor DNA. The blood, with its relatively high level of radioactivity, would provide a concentration gradient of iodinated bleomycin which would keep the binding sites on tumor DNA saturated and a slow rate of clearance of radioactivity from the tumor would be observed. In contrast, a tissue like muscle, with a low rate of DNA turnover would have low uptake of radioactivity and a rate of clearance which would directly reflect levels of radioactivity in the blood.

In all of the tissue distribution experiments performed in this study, the testes consistently showed an initial high uptake of radioactivity which was, subsequently, excreted rapidly like other non-tumor tissues. This is a deviation from observations made by others (3)(4)(5), that bleomycin did not reach high levels in the testes.

Two recent studies have reported the use of ^{131}I-bleomycin and ^{123}I-bleomycin (57)(58) in which very high specific activity iodinated bleomycin (greater than 100 mCi/mg) gave tumor:muscle and tumor:blood ratios of 36:1 and 12:1 at 6 hours after administration. This is in contrast to the values of 16:1 (tumor:muscle) and 8:1 (tumor:blood) obtained in this present study. However, the specific activity of ^{125}I-bleomycin A_2 and ^{125}I-bleomycin complex as reported in this thesis was relatively low, being in the order of 12-14 mCi/ml.

The anomalous uptake of radioactivity into the testes and the differences between the tumor:tissue ratios observed for the bleomycins iodinated to different levels of specific activity could possibly be explained by the results reported in a study involving the relationship between the specific activity of a product and its tissue distribution (98). This abstract suggested that with some radiopharmaceuticals there may be a relationship between the administered mass and the resultant target concentrations and therefore the law of mass action would dictate that as the binding sites of a tissue were saturated, maximum uptake would occur and excess would spill over into non-target areas. The resulting target to non-target ratios observed would be a function of the specific activity of the radiopharmaceutical (98). The low specific activity iodinated bleomycin would contain a high ratio of non-radioactive to radioactive bleomycin molecules. Sites of uptake in tissues would then tend to be occupied primarily by non-radioactive bleomycin. The absolute uptake of radioactivity observed in these tissues would be lower than if a high specific activity product were used. Once the binding sites on the tissues of primary uptake were saturated with the non-radioactive bleomycin, radioactive and non-radioactive bleomycin would tend to spill over into tissues of secondary uptake such as the testes.

A study, previously discussed (9) using ^{57}Co-bleomycin labeled to different specific activities has also documented this effect on tumor:muscle and tumor:blood ratios. Perhaps additional work would be warranted using the ICl method of iodination to produce much higher specific activity radioiodinated bleomycin than those used in this study. In addition, Iodine-123, with its 159 KeV gamma would be a better choice of radionuclide than the iodine-125 which was used throughout this project.

It is felt that since the tissue distribution pattern might be effected by the specific activity of the radiopharmaceutical being administered, it would not be completely valid to use data obtained from this study, using low specific activity ^{125}I-bleomycin, to predict the behavior of ^{123}I-bleomycin in humans. It is envisioned that the use of high specific activity ^{123}I-bleomycin would yield superior tumor:background ratios than those obtained in this present study.

F. Tissue Distribution of ^{114m}In-Bleomycin A_2 in Tumor-Bearing Mice

In order to further evaluate the affinity of ^{125}I-bleomycin for tumor tissue, a series of tumor-bearing mice were injected with ^{114m}In-bleomycin A_2. The indium-bleomycin was chosen as a standard for comparison since ^{111}In-bleomycin is used clinically for tumor imaging and is commercially available. The ^{114m}In was chosen as a labeling nuclide simply for the convenience of its long half-life.

The ^{114m}In-bleomycin A_2 was prepared after the method of Grove et al. (9). The radiopharmaceutical, as prepared was found to have a radiochemical purity of 93.6% and was used without further purification.

Tissue distribution studies were done using 6 tumor-bearing mice at 1, 6, 12, 24, 48 and 72 hour intervals after the intravenous administration of 30.3 uCi$^{(a)}$ in 0.1 ml of solution. The results are shown in Table 23 and Figures 15 and 16. The kidney showed

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(a) 30.3 uCi of radioactivity was 93.6% radiochemically pure and therefore 28.4 uCi of radioactivity would be associated with bleomycin A_2.

Table 23

Tissue Uptake[a][b] in Tumor-Bearing Mice

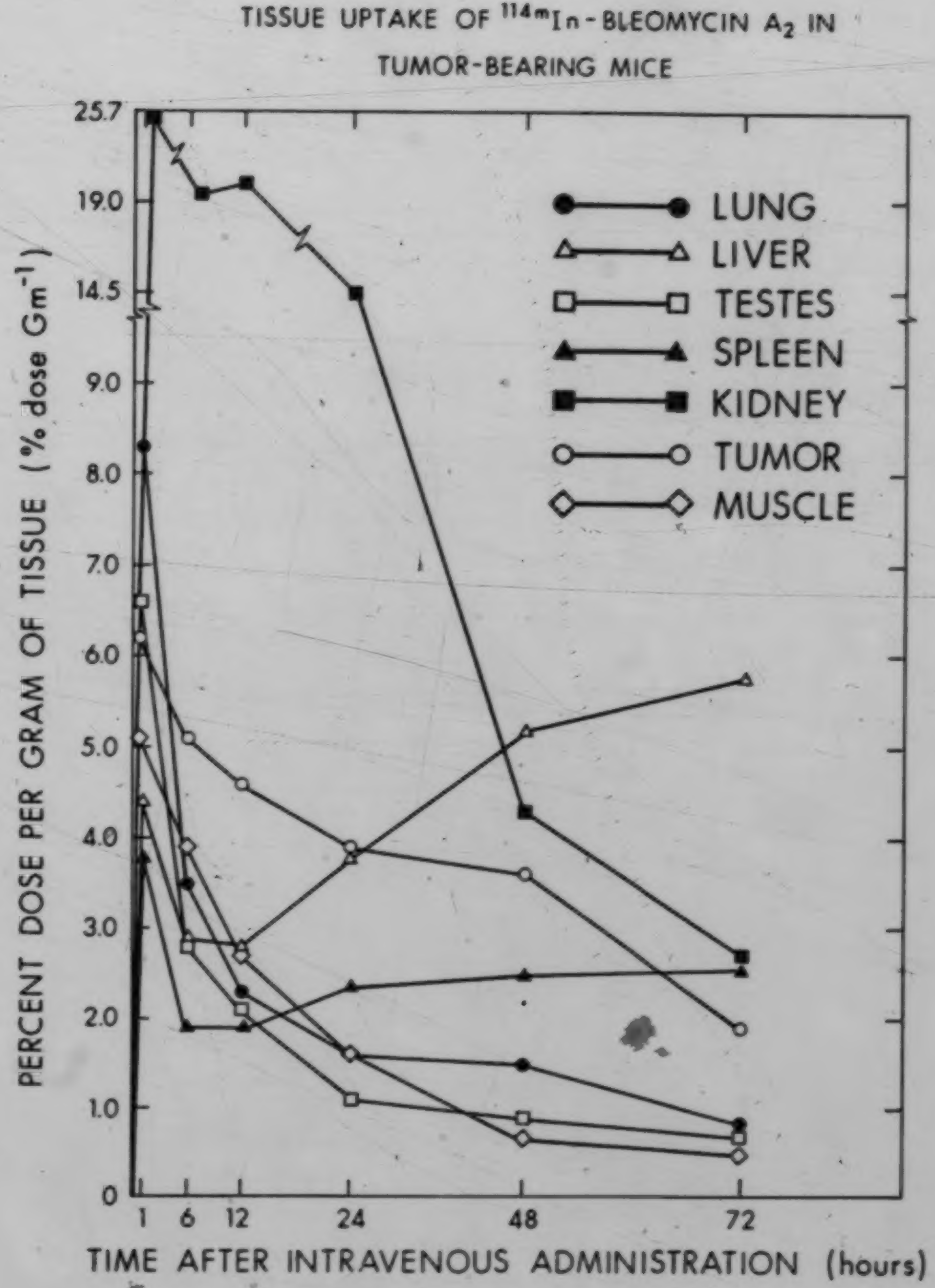
After Intravenous Administration of ^{114m}In-Bleomycin A_2

Tissue	Time (Hours) After Intravenous Administration					
	T1	T6	T12	T24	T48	T72
lung	8.27±1.29	3.52±0.94	2.27±0.42	1.58±0.32	1.48±0.45	1.13±0.21
liver	4.42±0.89	2.83±0.43	2.65±0.28	3.77±0.41	5.14±0.56	5.79±0.58
testes	6.57±1.19	3.30±0.51	2.11±0.32	0.11±0.02	0.09±0.02	0.08±0.02
spleen	3.81±0.31	1.86±0.21	1.91±0.16	2.39±0.46	2.52±0.41	2.63±0.35
kidney	25.70±3.55	19.12±2.58	19.20±1.15	14.52±1.60	14.3 ±1.33	2.71±0.52
tumor	6.22±0.69	5.05±1.22	4.55±0.71	3.92±0.27	3.58±0.33	1.93±0.28
muscle	5.09±0.61	3.93±0.13	2.71±0.32	1.56±0.30	0.65±0.13	0.45±0.18
blood[c]	4.91±0.31	2.28±0.19	1.87±0.41	1.13±0.11	0.33±0.06	0.20±0.04

[a] Expressed as percent of injected dose per gram of tissue. Mean of 6 animals ± standard deviation.

[b] All animals were injected with 0.1 ml of ^{114m}In-bleomycin A_2 solution (30.3 uCi).

[c] Expressed as percent of injected dose per 1.0 ml of whole blood. Mean of 6 animals ± standard deviation.



TISSUE UPTAKE OF ^{114m}In-BLEOMYCIN A_2 IN TUMOR-BEARING MICE

Figure 15

UPTAKE IN MUSCLE, TUMOR, LIVER AND BLOOD OF ^{114m}In-BLEOMYCIN A_2 IN TUMOR-BEARING MICE

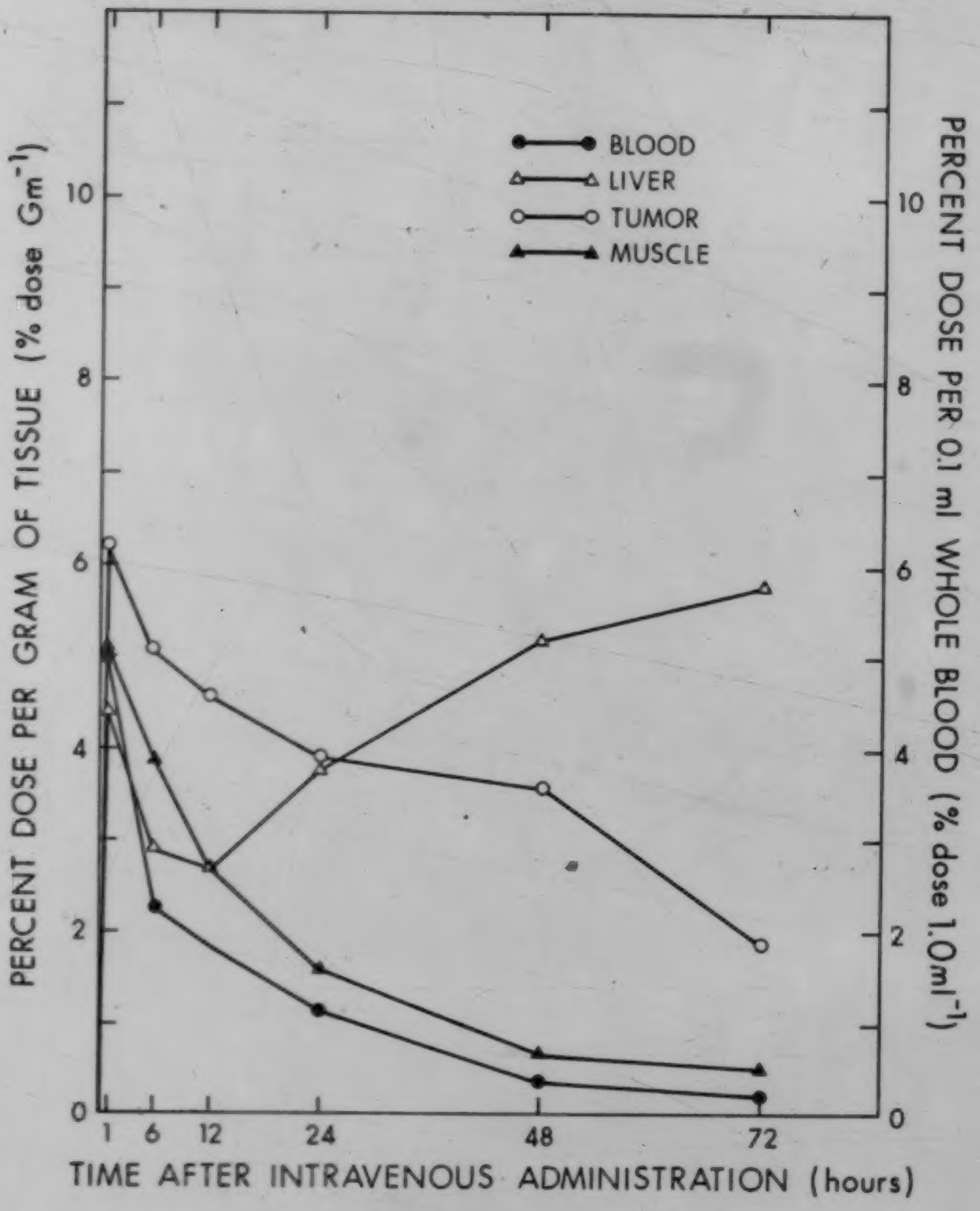


Figure 16

high levels of radioactivity throughout the period of the study while radioactivity in the blood fell rapidly, relative to other tissues. The liver and spleen both showed significant accumulation of radioactivity at 12 hours after administration. This uptake continued to increase indicating that free ^{114m}In was accumulating in these tissues presumably from the radiochemical impurities and also from the disruption of the chelate bond (10)(11). The maximum tumor:muscle and tumor:blood ratios of 5.5:1 and 10.8:1 respectively, occurred at 48 hours after injection. This is comparable to results reported in another study (14) which found tumor:muscle and tumor:blood ratios of 6:1 and 9:1 at 48 hours. Radioactivity was found to be rapidly excreted by most tissues with the exception of liver and spleen.

Therefore the ^{125}I-bleomycin A_2 gave higher tumor:muscle and tumor:blood ratios than ^{114m}In-bleomycin A_2 at all time intervals studied. The ^{125}I-bleomycin A_2 was rapidly cleared from all tissues and the whole body thus implying that radiation doses would be relatively low. In contrast, clearance of ^{114m}In-bleomycin A_2 was slow with significant quantities of radiation accumulating in the spleen and liver, thus implying a higher internally absorbed radiation dose to selected tissues and the whole body from this radiopharmaceutical.

G. Whole Body Excretion Analysis

In order to evaluate the rate of excretion of ^{125}I-bleomycin A_2 from the body, mice were administered 11.6 uCi intravenously in 0.1 ml of solution. Radiochemical purity, as determined on Eastman chromatograms, was 97% so that the 11.6 uCi of radioactivity contained about 0.4 uCi of free $Na^{125}I$ which would be trapped by the thyroid and would be expressed as a long term component of the excretion curve.

Five minutes after the intravenous administration of 0.1 ml of ^{125}I-bleomycin A_2, the total amount of radioactivity in each mouse was measured in a small animal whole body counter for a period of 10 seconds. The count obtained was considered as the standard initial 100% radioactivity of that mouse. All subsequent counts were corrected for decay and expressed as a percent of this initial value. The mice were assayed for radioactivity at 1, 6 and 12 hours after injection and then daily for a period of 10 days. The results obtained are shown in Table 24. Radioactivity was rapidly cleared from the body with less than 10% remaining 3 days after administration.

The excretion curve, Figure 17 was visually resolved into 2 components by extrapolating the straight portions of the curve back to their respective axis. The rapid component was found to have a half-life of about 0.6 days and represented about 90% of the initial radioactivity whereas the slow exponential excretion component had a half-life of about 6.2 days and represented 8-10% of the initial radioactivity.

The rapid clearance of radioactivity from the body seems to confirm the in vivo stability of the iodine-carbon bond since if dehalogenation occurred to any great extent, the level of radioactivity retained by the thyroid would increase and the long-lived excretion component would have an increasingly important influence on the nature of the excretion curve.

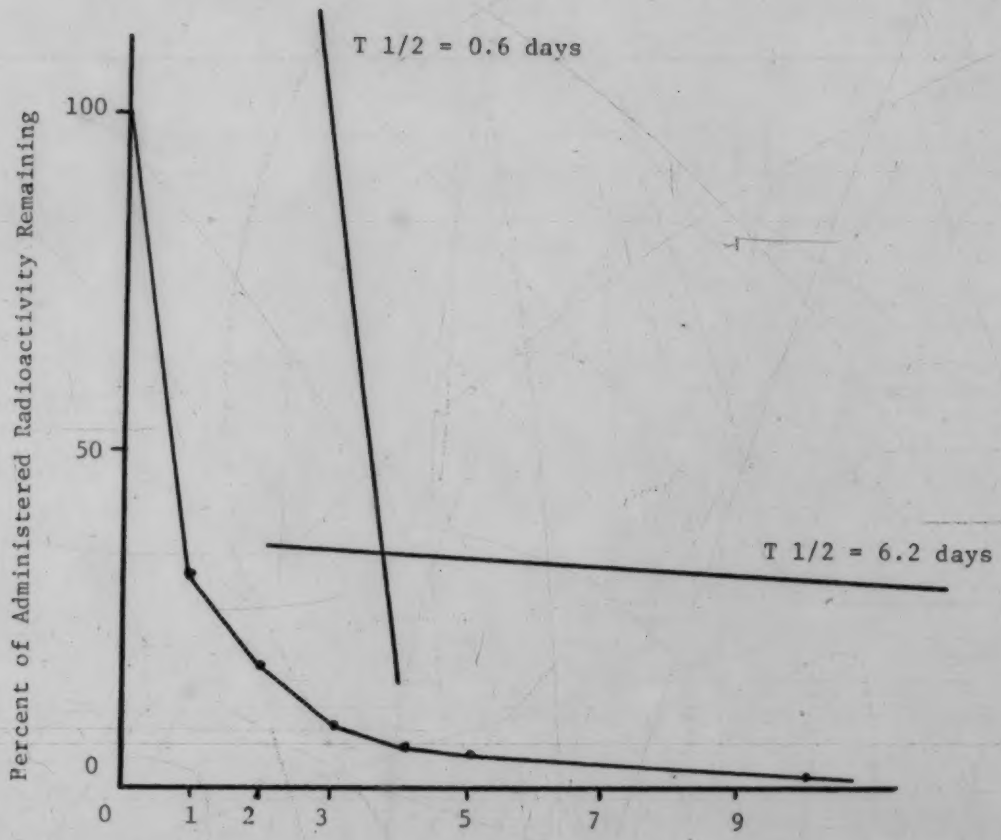
Table 24

Body Burden of ^{125}I in Mice Following Intravenous Injection of 11.6 uCi of ^{125}I-Bleomycin A_2 Solution[a]

Time After Administration (Days)		Percent of Dose Remaining[b]
Day 0	5 min.	100.0%
	1 hour	94.3±[illegible].4
	6 hours	79.9±5.2
	12 hours	58.7±3.1
1		18.5±1.9
3		9.4±1.4
4		6.1±1.8
5		3.8±0.9
6		2.1±0.6
7		1.9±0.6
8		1.7±0.4
9		1.6±0.2
10		1.6±0.3

(a) Determined by whole body counting.

(b) Mean of 3 mice ± standard deviation.



Time After Intravenous Administration (Days)

Compartmental Distribution of Whole Body Burden Curve of ^{125}I After Administration of ^{125}I-Bleomycin A_2

Figure 17

SUMMARY AND CONCLUSIONS

1. A method was developed for isolating and purifying the bleomycin A_2 fraction from the commercially available complex (Blenoxane, Bristol Labs.). The overall yield of bleomycin A_2 obtained from bleomycin complex was 35%.
2. The chloramine-T method of iodinating bleomycin was found to be unsatisfactory because of low radioiodination yields.
3. Bleomycin, as the A_2 fraction or as the complex, could be rapidly radioiodinated to high yields and specific activities using the ICl method. No noticeable changes in the chromatographic behavior of the iodinated bleomycins were observed indicating that the structure of the molecule was not adversely effected by the reaction conditions.
4. It was found that thin layers of Dowex 1x4 anion exchange resin could be used to remove any free $Na^{125}I$ remaining in the iodination reaction mixtures. The resulting iodinated bleomycin solution consistently had a radiochemical purity of greater than 95%.
5. A rapid, reliable method of assaying radioiodination reaction mixtures for yield and for radiochemical purity was developed using the Gelman ITLC chromatographic medium and 95% ethanol as solvent. The results obtained were reproducible and comparable to those obtained using alternative, more time consuming assay methods.
6. Radioiodinated bleomycins, in aqueous solution, were found to be stable in vitro to temperatures up to 100°C for 1 hour and to the presence of excess Cu^{+2}. The loss of radiolabel from iodinated bleomycin solutions was about 0.7% per day at 4°C, 1% per day at

20°C and 12-14% when exposed to 100°C for a period of 1 hour.

7. There were no significant differences in the tissue distribution of ^{125}I-bleomycin complex and ^{125}I-bleomycin A_2 in normal mice or in tumor-bearing mice. Initial uptake of radioactivity by the tumor was high and the rate of clearance was found to be slow, relative to muscle and other tissues.
8. Maximum tumor:muscle and tumor:blood ratios of about 16:1 and 8:1 were observed at 6 hours after administration for both ^{125}I-bleomycin A_2 and ^{125}I-bleomycin complex.
9. The analysis of the whole body burden of radioactivity after the intravenous administration into mice of ^{125}I-bleomycin A_2 indicated that the excretion curve could be resolved into 2 components with half-times of about 0.6 days and 6.2 days which represented 90% and 10% of the administered dose, respectively. Only 34% of the initial dose was detected at 24 hours indicating rapid clearance of the radioactivity from the body and no significant activity in the thyroid.
10. In-114m-bleomycin A_2 was found, for the Ehrlich's ascites tumor model in mice, to give maximum tumor:muscle and tumor:blood ratios of 5.5:1 and 10.8:1 respectively, at 48 hours after administration.
11. Radioiodinated bleomycin was found to give a higher tumor:muscle ratio than ^{114m}In-bleomycin A_2 in the mouse tumor model studied. In addition the radioiodinated bleomycin reached its maximum tumor:muscle ratio at 6 hours whereas ^{114m}In-bleomycin required 48 hours to reach its maximum value. Radioiodinated bleomycin

was quickly cleared from the body while the clearance of ^{114m}In-bleomycin tended to be slow.

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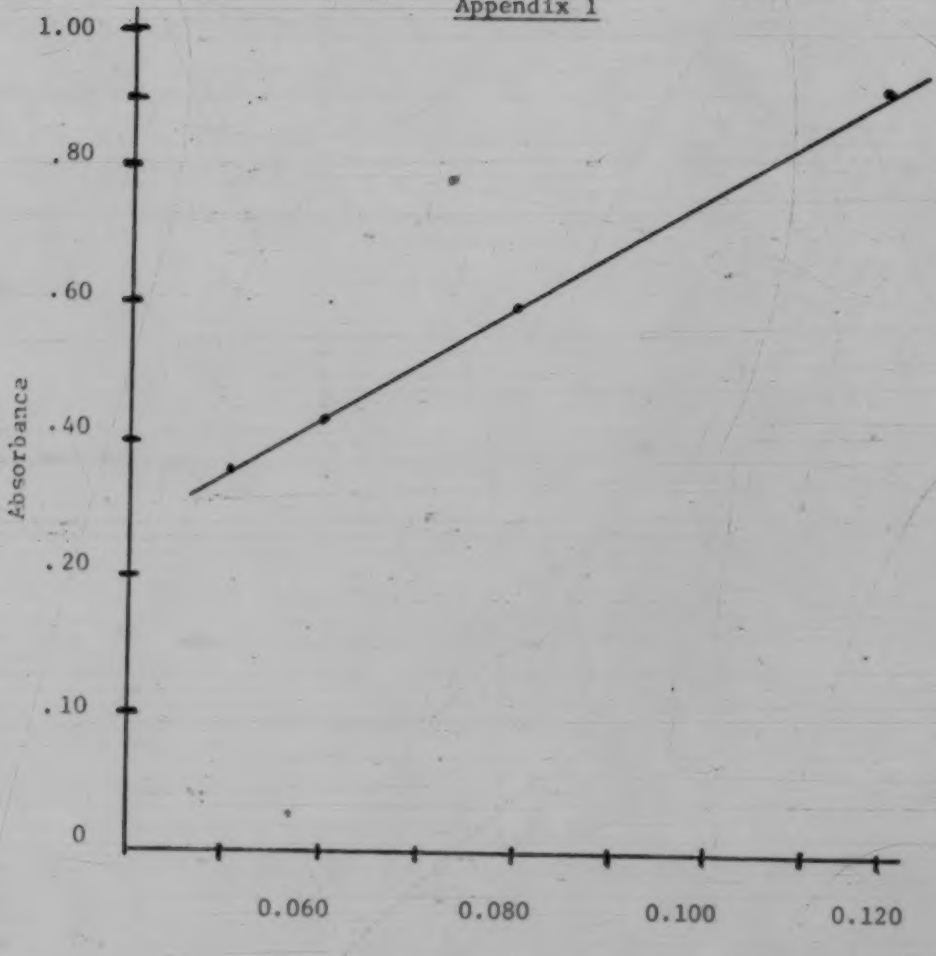
APPENDICES

Appendix 1



Concentration in mg/ml.

Standard Curve For Bleomycin A_2 in Aqueous Solution at 254 NM.

Appendix 2

Distribution of Na^{125}I(a) on Eastman(b) and Gelman(c) Chromatograms

Using 95% Ethanol as Solvent

System	Chromatogram Segment Number(d)						
	1	2	3	4	5	6	7
Gelman	2.00±0.10	0.50±0.10	0.50±0.10	0,60±0.10	10.53±2.10	35.61±3.40	50.50±4.60
Eastman	1.90±0.10	1.20±0.10	1.20±0.10	1.40±0.10	1.7 ±0.10	1.7 ±0.10	1.3 ±0.10

	Chromatogram Segment Number					
	8	9	10	11	12	13
Gelman	-	-	-	-	-	-
Eastman	2.0 ±0.10	3.8 ±0.13	26.51±3.65	40.27±5.19	13.85±2.3	3.1 ±1.7

(a) Expressed as a percentage of total radioactivity (in CPM) on chromatograms. Expressed as mean of 3 chromatograms ± standard deviation.

(b) Eastman chromatogram silica gel sheets #6061 (Eastman Kodak Co., Rochester, N.Y.)

(c) Gelman I.T.L.C. S.G. Type sheets (Gelman Instrument Co., Ann Arbor, Mich.)

(d) Each segment is 1 cm in length.

Appendix 3

Stability of ^{125}I-Bleomycin Complex - Chloramine-T

Day	20°C A (a)(b)(c)	20°C B	20°C C	4°C A	4°C B	4°C C
1	86.5±1.2	95.6±0.5	74.3±1.1	86.5±2.6	95.6±0.8	74.3±0.2
2	85.1±1.1	92.1±0.3	74.1±1.2	85.2±0.8	95.1±0.8	74.1±0.6
3	84.9±0.6	92.2±1.1	70.9±0.8	84.3±1.1	94.6±0.6	73.2±0.8
4	84.7±0.8	90.6±2.3	70.4±0.9	84.1±0.6	93.8±0.9	71.9±1.3
5	82.4±1.2	89.2±0.8	68.8±1.9	83.7±0.7	92.1±1.1	70.6±0.7
6	80.1±2.1	89.3±1.2	67.4±1.1	82.8±1.1	93.1±0.8	70.1±0.6
7	79.6±0.8	86.5±1.1	67.1±1.6	82.4±0.8	90.6±1.1	69.3±0.8
8	77.4±2.3	84.2±0.8	67.2±1.1	80.6±0.8	89.8±0.8	68.9±1.1
9	74.3±1.2	83.9±0.5	66.3±0.5	80.1±0.7	88.3±1.2	68.1±0.9
10	73.2±	82.3±1.1	66.1±0.6	79.4±1.1	87.6±1.1	68.7±1.1
20	64.8	70.6±0.5	53.8±0.1	73.5±0.8	80.6±1.1	63.8±0.8
% label lost	21.7	25	20.5	13	15	10.5
% lost/day	1.1	1.3	1.0	0.7	0.8	0.5

[a] Yields expressed as mean of 2 ± standard deviation.

[b] 20 ul aliquots chromatographed on Kodak #6061.

[c] Yields co-rected for background and physical decay.

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Appendix 3 (continued)

Stability of ^{125}I-Bleomycin A_2 - ICl Method

Day	20°C			4°C		
	A	B	C	A	B	C
1	93.2±1.6[(a)(b)(c)]	95.6±0.7	91.1±0.4	93.2±1.6	95.6±0.7	91.1±0.4
2	91.4±1.1	92.9±1.3	87.8±1.1	90.8±0.8	87.6±0.8	90.5±1.1
3	91.2±1.1	90.6±2.2	86.2±0.4	90.1±0.4	87.5±0.8	88.3±0.6
4	88.8±0.6	91.9±1.1	84.3±1.2	88.2±1.1	87.1±0.4	88.4±0.6
5	87.1±1.1	90.4±1.5	84.0±0.7	88.1±0.4	86.5±1.1	86.1±0.4
6	86.4±1.9	90.1±1.9	83.7±0.4	87.7±0.5	86.7±0.9	85.8±0.7
7	85.9±0.9	88.9±0.6	82.9±1.1	86.9±0.3	85.1±1.3	85.2±1.1
8	85.4±1.7	88.3±1.2	82.3±0.8	87.1±0.9	84.6±0.8	86.3±1.2
9	85.1±1.1	85.3±1.6	81.1±1.1	86.0±0.8	82.4±0.8	85.1±0.6
10	81.9±1.3	83.2±0.4	80.9±0.6	85.4±0.8	82.2±0.4	86.2±1.4
20	75.2±2.1	77.4±1.1	69.0±1.1	82.3±0.6	79.1±1.1	79.8±1.7
30	64.8±2.3	67.1±0.9	63.3±1.2	80.1±1.1	77.2±1.7	76.9±0.4
% label lost	28.4	28.5	27.8	13.1	18.4	14.2
% lost/day	0.95	0.95	0.93	0.44	0.61	0.47

[(a)] Yields expressed as mean of 2 standard deviation.

[(b)] 10 ul aliquots of sample chromatographed on Eastman #6061.

[(c)] Yields corrected for background and physical decay.

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Appendix 3 (continued)

Stability of ^{125}I-Bleomycin A_2, Produced by the Chloramine-T Method, at 4°C and 20°C[a]

Percent Label Remaining[b]

Day	4°C	20°C
1	100.0	100.0
2	99.2±0.6	98.1±1.7
3	98.3±0.8	96.7±1.4
4	97.4±0.7	95.8±1.8
5	96.0±0.9	93.7±1.4
6	95.8±1.6	91.0±3.5
7	94.5±1.0	90.9±0.9
8	93.3±0.6	89.3±1.2
9	92.2±0.5	87.6±1.7
10	91.9±0.5	86.6±2.2
20	86.6±2.0	73.7±1.3
Total label lost	13.4	26.3
% label lost/day	0.7	1.3

(a) Assay carried out on Eastman #6061 using 95% ethanol as solvent system. All values corrected for background and physical decay.

(b) Expressed as mean of 3 values ± standard deviation.

Appendix 4

Activity of $Na^{125}I$ Required to Give a Theoretical 100% Labeling of 150 ug of Bleomycin A_2

1. Number of molecules of bleomycin A_2 in 150 ug, assuming a molecular weight of 1500,

$$= \text{Avogadro's Number X molarity}$$

$$= 6.023\text{x}10^{23} \text{ x } \frac{1.50\text{x}10^{-4}}{1500}$$

$$= 6.023\text{x}10^{23} \text{ x } 10^{-7}$$

$$= 6.023\text{x}10^{16} \text{ molecules of bleomycin } A_2$$

2. Number of atons of $Na^{125}I$/mCi, assuming a half-life of $Na^{125}I$ of 58 days and an effective half-life of 1.443,

= number of disintegrations/mCi/min x minutes/hr. x hours/day x half life x effective half life

$$= 2.22\text{x}10^{9}\text{x}60\text{x}24\text{x}58\text{x}1.443$$

$$= 2.68\text{x}10^{5+9}$$

$$= 2.68\text{x}10^{14} \text{ atoms of } Na^{125}I \text{ per mCi}$$

3. Number of mCi of $Na^{125}I$ required for a 1:1 molar ratio with $6.023\text{x}10^{16}$ molecules of bleomycin A_2

$$= \frac{\text{number of bleomycin } A_2 \text{ molecules in 150 ug}}{\text{number of atoms in a mCi}}$$

$$= \frac{6.023\text{x}10^{16}}{2.68\text{x}10^{14}}$$

$$= 225 \text{ mCi}$$

Appendix 5

Calculation of Number ^{125}I Atoms Incorporated Per Molecule of Bleomycin, Assuming 2.5 mCi of $Na^{125}I$ in the Reaction Mixture and 80% Labeling Efficiency

(a) Number atoms of ^{125}I in 2.5 mCi of $Na^{125}I$

$= 2.5 \times 2.22 \times 10^{9} \times 60 \times 24 \times 58 \times 1.443$

$= 6.69 \times 10^{14}$ atoms of ^{125}I

assuming 80% labeling efficiency, then $6.69 \times 10^{14} \times 8.0 \times 10^{-1}$

$= 5.35 \times 10^{14}$ atoms of ^{125}I would be incorporated into a total of 6.02×10^{16} molecules of bleomycin or

8.9×10^{-3} atoms of ^{125}I/molecule of bleomycin A_2

(b) The specific activity attained under these conditions was

$$= \frac{2500 \text{ uCi x labeling efficiency}}{150 \text{ ug bleomycin } A_2}$$

$= 13.3$ uCi/ug of ^{125}I-bleomycin A_2

Appendix 6

Preliminary Tissue Distribution in Tumor-Bearing Mice
After Intravenous Administration of 20 uCi of ^{125}I-Bleomycin Complex(a)

Time (Hours)	Tissue CPM/mg					
	lung	liver	testes	spleen	kidney	muscle
1.5	172±20	70± 9	191±26	45± 6	666±91	84± 1
3.0	89±14	25± 4	142±19	14± 2	253±26	34± 4
6.0	10± 2	14± 2	69±12	8± 1	182±18	11± 2

	Blood(b)	Brain	Gut(c)	Bone(d)	Heart(e)	Skin(f)	Tumor
1.5	39516±627	29± 3	51±11	84± 9	35± 4	273±29	235±27
3.0	3996±247	9± 2	23± 5	35± 5	19± 3	168±20	209±23
6.0	888±102	3± 0	13± 3	19± 2	29± 2	114±14	63±11

(a) Expressed as mean of 3 animals ± standard deviation.

(b) CPM per 0.1 ml whole blood.

(c) Mid section of gut.

(d) Femor.

(e) Perfused with normal saline.

(f) From dorsal region of animal.

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Appendix 6 (continued)

Preliminary Tissue Distribution of ^{125}I-Bleomycin Complex in Tumor-Bearing Mice After Intravenous Administration

Time (hours)	Residual Radioactivity in Carcass (cpm)		
	Tail	Trunk	Head
1.5	67750±4327	1.3x10^{6}±116000	180000±11419
3.0	45122±4091	638100±54412	127641±9612
6.0	17981±1089	186018±14110	49618±5112